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β-Carbolines That Accumulate in Human Tissues May Serve a Protective Role against Oxidative Stress*

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β-Carbolines are tricyclic nitrogen heterocycles formed in plants and animals as Maillard reaction products between amino acids and reducing sugars or aldehydes. They are being detected increasingly in human tissues, and their physiological roles need to be understood. Two β-carboline carboxylates have been reported to accumulate in the human eye lens. We report here on the identification of another β-carboline, namely 1-methyl-1-vinyl -2,3,4-trihydro-β-carboline-3-carboxylic acid, in the lenses of some cataract patients from India. Analysis of these three lenticular β-carbolines using photodynamic and antioxidant assays shows all of them to be inert as sensitizers and effective as antioxidants; they quench singlet oxygen, superoxide and hydroxyl radicals and inhibit the oxidative formation of higher molecular weight aggregates of the test protein, eye lens γ-crystallin. Such antioxidative ability of β-carbolines is of particular relevance to the lens, which faces continual photic and oxidative stress. The β-carboline diacid IV is also seen to display an unexpected ability of inhibiting the thermal coagulation of γ-crystallin and the dithiothreitol-induced precipitation of insulin. These results offer experimental support to earlier suggestions that one of the roles that the β-carbolines have is to offer protection against oxidative stress to the human tissues where they accumulate.

β-Carbolines are tricyclic, heterocyclic alkaloids that are formed by the Maillard reaction through the condensation of reducing sugars and aldehydes such as glucose or acetaldehyde with compounds possessing a free amino group, such as amino acids (1–4). β-Carbolines formed between hexulose, xylose, furan-2-carboxaldehyde, acetaldehyde, and glucose on the one hand and amino acids such as lysine, glycine, proline, arginine, histidine, and tryptophan on the other have been reported (5–10). They are formed endogenously in plants and animals (for example, harmane, structure **I** in Fig. 1, is isolated from the bark of *Sickingia rubra*, whereas harmine (**II**), harmaline (**IIa**), and harmalol (**IIb**) occur in the seeds of *Peganum harmala*), but their physiological role is not clear. Some of them are mildly psychoactive (6). The prototypic β-carboline, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (compound **III** in Fig. 1), detected in some food items, is implicated as a precursor of mutagenic *N*-nitroso compounds (7); **III** has also been identified as the possible causative substance of eosinophilia-myalgic syndrome associated with the ingestion of L-Trp in mammals (8–10). Some of the β-carbolines have been shown to be phototoxic to bacteria and insects (11), and attempts have been made to correlate their phototoxicity with their ability to produce reactive oxygen species upon irradiation, *i.e.* photodynamic or sensitizer abilities (11–13). On the other hand, there have been reports (14–16) that some of these Maillard products are antioxidative in nature. β-Carbolines are found ubiquitously in a variety of foods: grain flour, soy sauce and soy protein, milk, beer, and wine, and perhaps as a consequence, in animal fluids and tissues such as blood, milk, urine, kidney, liver, and brain (10, 17). Several reports have appeared in recent literature of the presence of β-carbolines in human tissues. It thus becomes important to understand their physiological role in human health.

Manabe *et al*. (18) have shown that **III** accumulates steadily in the eye lens with age and in significantly higher amounts in senile cataract and diabetic cataract lenses. Dillon *et al*. (19) have discussed the presence of the β-carboline diacid **IV** in the lens. In this paper we report on the presence of yet another β-carboline (compound **V** in Fig. 1) from the water-soluble portion of human cataract lenses. The physiological role of the β-carbolines that accumulate in the human lens is of particular ophthalmological interest; do they play a protective role by filtering the UVA and UVB radiation from the vitreous and retina? Are they benign accumulants, or do they behave as potentially harmful agents through possible photodynamic action that would impose oxidative stress? This question is relevant because many other Trp metabolites and derivatives are also known to accumulate in the lens, some of which seem to be photodynamic, *e.g. N*-formylkynurenine (20) or 4-hydroxyquinoline carboxylate (21), whereas others such as 3-hydroxykynurenine might offer antioxidant protection (22).

With this question in mind we have investigated the photobiological and antioxidant properties of a series of β-carboline compounds. Our results suggest that the β-carbolines that accumulate in human tissues, namely **III**, **IV**, and **V**, have little or no photodynamic properties but display a possible antioxidant, protective role. In addition, the β-carboline diacid **IV** that has been shown to be in the lens, is also seen to display a surprising ability of solubilizing or inhibiting the precipitation of proteins.

MATERIALS AND METHODS

Isolation of **V**

Human lenses excised through cataract surgery of elderly patients, obtained from the rural eye camps in the state of Andhra Pradesh in India, were homogenized in 80% alcohol and centrifuged at 10,000 × *g*

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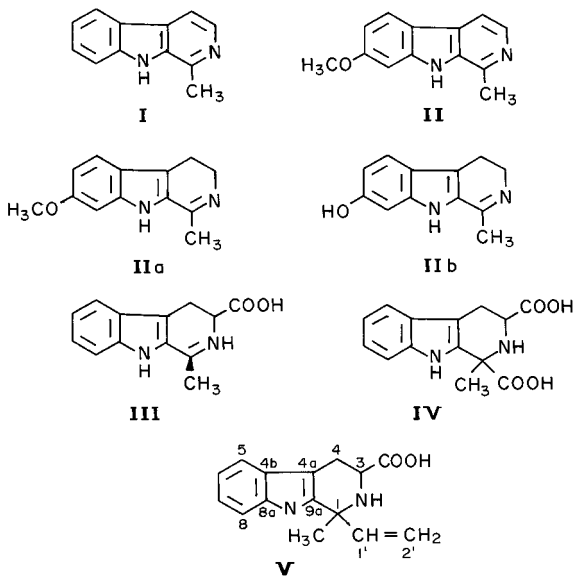

FIG. 1. **Structures of some β-carbolines. I**, harmane; **II**, harmine; **III**, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid; **IV**, 1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid; and **V**, 1-methyl-1-vinyl-2,3,4-trihydro-β-carboline-3-carboxylic acid.

for 15 min at 4 °C. The resulting supernatant solution was collected separately and chromatographed on a C_{18} reverse phase column (20 × 1.5-cm inner diameter) using a Hewlett-Packard HPLC[1] system. The mobile phase consisted of a 0–100% gradient system of 0.1% trifluoroacetic acid, in 1:1 CH_3CN:trifluoroacetic acid (0.1%) run over for 32 min. The flow rate was kept at 0.4 ml/min, and the absorbance was monitored at 280 nm. Several eluates absorbing at 280 nm were detected at retention times of 6.8, 8.6, 9.4, 14.6, 19.0, and 27.3 min. The eluate at 27.3 min was rechromatographed on the same system and purified to homogeneity to get about 2 mg of the compound. Analysis of the other eluates by co-HPLC with authentic standards established them to be kynurenines and xanthurenic acid derivatives.

*Synthesis of **III***

The synthesis followed the procedure described earlier (23) and is called the Pictet-Spengler reaction between the amino acid and the aldehyde via the Schiff base and cyclization to a tetrahydro-β-carboline. Both the (1*S*,3*S*) and (1*R*,3*S*) diastereoisomers are obtained in the reaction, which are easily separated and isolated in the pure form. Of the two, the *SS* isomer has been seen to be present in amounts four times higher than the *RS* isomer (7, 18).

A mixture of 2 g (0.245 mol) of L-Trp, 1.6 ml (0.84 mol) of freshly distilled acetaldehyde, and 1 ml of 0.1 N H_2SO_4 was dissolved in 30 ml of water and stirred under N_2 atmosphere at room temperature (about 25 °C) for 8 h. The precipitated material was filtered and recrystallized from water to yield 1.3 g (75% yield) of pure product, melting at 293 °C. Mass, NMR and optical spectra confirmed its identity as (−)(1*S*, 3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid or the *SS* isomer of **III**.

The mother liquor was evaporated to dryness, 7.8 g (0.157 mol) of acetaldehyde and 10 ml of 0.1 N HCl added, and the mixture was diluted using 10 ml of water and stirred under an N_2 atmosphere at room temperature overnight. The resultant precipitate was collected and recrystallized from water to yield 2 g (4.5% yield) of the final product, m.p. 242–244 °C. Spectral characterization as above confirmed its identity as (1*R*,3*S*) **III**. We have thus isolated both the *SS* and *RS* isomers, studied them, and find that they have essentially the same photobiological and antioxidant properties. In light of the fact that the *SS* isomer is the major component in biological samples we present the results of this isomer, abbreviated as **III** in this paper.

*Synthesis of the Diacid (**IV**)*

2.5 g of L-Trp was dissolved in 20 ml of water with warming. About 4 ml of freshly distilled pyruvic acid was added to the warmed solution, and then the mixture was cooled to 30 °C and kept aside. The condensation product separated as long needles the next day. The yield of crystals after filtration, washing with water, and drying in a desiccator was 1.7 g. After concentration of the mother liquor in vacuum, 0.42 g of the substance separated. To recrystallize, the compound was dissolved in water with slight warming and the solution concentrated under vacuum till the crystallization started (yield 2.12 g).

*Synthesis of Harmane (**I**)*

To a boiling solution of **III** (0.25 g) in 65 ml of water, 12.5 ml of potassium dichromate solution was added followed by 2.5 ml of acetic acid. The solution was boiled for 1 min, cooled, and treated with dilute sodium sulfite solution to remove the excess oxidizing agent, made strongly alkaline with sodium carbonate, and shaken with ether. The ether extract was collected and evaporated to dryness under reduced pressure to yield harmane (0.152 g). Recrystallization with alcohol gave yellow needles, m.p. 238 °C.

*Synthesis of Harmine (**II**)*

Harmine was prepared following a procedure similar to the method mentioned for the preparation of harmane. Recrystallization was carried out in methanol-water to get a yield of 0.042 g.

Spectral Measurements

Optical absorption spectra were recorded using a Hitachi model U-2000 spectrometer. NMR spectra were recorded using a Bruker AC 300F (300 MHz) instrument. Mass spectra were recorded using a Va 7070-H EI (Va Micromass, U. K.) and a Hewelett-Packard series 1100 MSD mass spectrometer. Fourier transform infrared (FTIR) spectra were measured using a Nicolet (Impact 400) FTIR spectrometer. Fluorescence spectra were recorded in the steady-state mode using a Hitachi model F-4000 spectrofluorometer. The fluorescence quantum yields were determined using the procedures described earlier (24), using the relationship $Q_x = Q_s\,(F_x/F_s)\,(A_s/A_x)$ where the subscripts s and x refer to the standard compound (L-Trp, $Q_s = 0.13$) and the sample, respectively. *F* is the wave number-integrated area of the corrected emission at constant slit openings and *A* the absorbance at the excitation wavelength (always less than 0.1, so as to avoid the inner filter effect). We calculated the area of the corrected emission spectrum using the built-in computer of the spectrofluorometer. Fluorescence lifetime measurements were done at the CCMB using a Photon Technology instrument and also at the Tata Institute of Fundamental Research, Mumbai, using a home-built instrument in the laboratory of Professor N. Periasamy (25), whom we thank for help with the measurements. Solutions were freshly prepared in water before use.

Photodynamic Assay

Singlet Oxygen Detection—$^1O_2$ was detected by the method developed by Kraljic and Mohsni (26). Each of the test compounds was exposed to light at its absorption maximum (273 nm in the case of **III**, **IV** and **V** and 330 nm in the case of **I** and **II**) in the presence of 10 mM imidazole and 50 μM *N*,*N*-dimethyl-*p*-nitrosoaniline, or RNO, in 50 mM phosphate buffer, pH 7.4, for a chosen period of time. The light source was the 450-W xenon arc lamp of the Hitachi spectrofluorometer, and the light flux was estimated by actinometry in a separate earlier experiment to be 0.2 mW/cm^2, 10^{14} photons/s. Any $^1O_2$ that is generated by photoexcitation of the molecule reacts with imidazole to form a transannular peroxide that bleaches RNO, and the bleaching is monitored spectrophotometrically at 440 nm.

Superoxide Detection—$O_2^{\cdot-}$ was detected using the superoxide dismutase-inhibitable cytochrome *c* reduction method (27). Anthranilic acid (3 mM) was used as the sensitizer to generate $O_2^{\cdot-}$, and the samples containing 20 μM ferricytochrome c in 20 mM phosphate buffer, pH 7.4, were illuminated at 330 nm in the absence and presence of test compounds, in the instrument and under the conditions described above. The reduction of ferricytochrome *c* was monitored spectrophotometrically at 550 nm, using $\epsilon_{500} = 20{,}000\ M^{-1}\ cm^{-1}$ for the reduced-oxidized cytochrome *c* (28).

ABTS Antioxidant Assay

The assay was performed following the procedure by Miller *et al.* (29, 30). Briefly, when azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS is incubated with a peroxidase (such as metmyoglobin) and hydrogen peroxide, the relatively long lived radical cation, $ABTS^{\cdot+}$, is formed. When the peroxidase is metmyoglobin, the $ABTS^{\cdot+}$ radical cation that forms upon intercalation with ferryl myoglobin has absorption maxima at 650, 734, and 820 nm. In the presence of antioxidant

[1] The abbreviations used are: HPLC, high performance liquid chromatography; FTIR, Fourier transform infrared; RNO, *N*,*N*-dimethyl-*p*-nitrosoaniline; ABTS, azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).

reductants or hydrogen donors, the absorption of this radical cation is quenched to an extent that can be related directly to the antioxidant capacity of the added substance.

ABTS (30 μl, 5 mM), 50 μl of metmyoglobin (50 μM), and 820 μl of phosphate buffer (50 mM, pH 7.4) (of which 5 μl, or the desired volume, was replaced when a sample was being investigated) were mixed, and the reaction was initiated by the addition of 100 μl of hydrogen peroxide (1 mM). The absorbance at 734 nm (a beautiful bluish green color) was measured as a function of time at 5-min intervals for 20 min.

Protein Studies: Isolation and Purification of γ-Crystallin

The test protein, bovine eye lens γ-crystallin, was isolated in the laboratory by the following procedure. Fresh calf lenses were homogenized in Tris-HCl buffer, pH 7.2, containing 100 mM NaCl, 1 mM EDTA, and 0.02% sodium azide and centrifuged at 5,000 × *g* at 4 °C for 20 min. The soluble proteins in the supernatant were fractionated by gel filtration on a column of Bio-Gel A-1.5m (1.8 × 180 cm) at 4 °C. The fractions corresponding to β_H-, β_L-, and γ-crystallins were pooled and dialyzed against water. Further, the fractions corresponding to γ-crystallin were pooled and concentrated at 4 °C by ultrafiltration using an Amicon ultrafiltration unit and were stored at 4 °C until used for the experiment.

γ-Crystallin is known to aggregate and precipitate upon irradiation at 295 nm (photoaggregation) (31) as well as upon heating (thermal aggregation) (32). To study the effect of the β-carbolines on the photoaggregation behavior of γ-crystallin, the protein solutions, with or without the additive, were irradiated at 295 nm for fixed time periods with an excitation bandpass of 20 nm using the Hitachi F-4000 spectrofluorometer. After each time period of irradiation, the excitation and emission monochromators were both set to 600 nm with the excitation and emission bandpass 5 and 3 nm, respectively, to measure the relative scattering. The relative scattering was plotted against irradiated time. The concentration of γ-crystallin was 1 mg/ml. As a control, the same scattering test was performed while irradiating the solution at 350 nm (where the protein does not absorb) under similar conditions. No scattering was displayed in this case.

Covalent Cross-linking of γ-Crystallin: SDS-Polyacrylamide Gel Electrophoretic Analysis

Photodynamic Method—γ-Crystallin (1 mg/ml) was irradiated in a quartz cuvette in the presence of riboflavin as externally added sensitizer and the test carboline. The samples were irradiated at 446 nm under constant stirring for 30 min using the 450-W xenon arc lamp in the Hitachi spectrofluorometer. All samples were then reserved for polyacrylamide gel electrophoresis, using 10% acrylamide for setting the gel, and in the presence of 10% SDS and β-mercaptoethanol.

Fenton Reaction Method—The same experiment was done here, except that instead of using a sensitizer and light, the OH˙ radical was produced via the Fenton reaction using $FeCl_3$ (10 mM) and ascorbic acid (0.1 M) in the dark at room temperature (33). The protein solutions were incubated in the Fenton mixture for up to 72 h and then analyzed using SDS-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol as above.

Thermal Aggregation of γ-Crystallin

Thermal aggregation of γ-crystallin was monitored by the following procedure. γ-Crystallin (0.5 mg/ml) in 20 mM phosphate buffer, pH 7.4, was equilibrated at the required temperature for 5 min with constant stirring in the sample holder. Then, the extent of aggregation was measured as a function of time by the scattering of 600 nm light, by setting the excitation and emission bandpasses at 5 and 3 nm, respectively. In other experiments, the buffer containing 0.5 mg/ml γ-crystallin was equilibrated at the required temperature. Different stock solutions containing β-carbolines were then added and aggregation measured as described above.

Dithiothreitol-induced Aggregation of Insulin

Insulin at a concentration of 0.2 mg/ml (in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl) with or without IV was equilibrated at 42 °C for 5 min with constant stirring in the sample holder using a Julabo thermostatted water bath. The reduction of insulin was initiated by adding 20 μl of 1 M dithiothreitol to 1 ml of the sample, and the extent of aggregation of the insulin B chain was measured as a function of time, by monitoring the scattering of 465 nm light in a Hitachi F-4000 fluorescence spectrophotometer. The excitation and emission monochromators were set at 465 nm with bandpasses of 1.5 nm.

Circular Dichroism (CD) Measurements

CD spectra were recorded using a Jasco J-715 spectropolarometer. CD spectra of γ-crystallin at different temperatures were recorded using water-jacketed cuvettes. The required temperatures were maintained using a Julabo circulating water bath. Far UV CD spectra were recorded using a 1-mm path length cell. A concentration of 0.5 mg/ml γ-crystallin was used for CD studies.

Electronic Energy Level Calculations

The molecular orbital energy levels of **I**, **III**, **IV**, and **V** were computed using the semiempirical method of Dewar *et al.* (34), using the program package MOPAC 93 (Fujitsu Inc.). Optimization was performed using the "Precise" criteria and including configuration interaction scheme; all excitations were within the eight molecular orbitals bracketing the HOMO- LUMO 4900 microstates, in the laboratory of Professor T. P. Radhakrishnan of the University of Hyderabad.

RESULTS AND DISCUSSION

A New β-Carboline from the Human Cataract Lens—Two β-carbolines, namely **III** and the diacid **IV**, have been identified earlier as present in the human lens (18, 19). Here we report on the presence of another new member of the β-carboline family from the 80% ethanol extract of human lenses. Because it is extracted in ethanol, it occurs in the lens in the free form and not in the protein-bound form. HPLC separation of the extract yielded fractions with retention times of 6.8, 8.6, 9.4, 14.6, 19.0, and 27.3 min. Analysis of the eluates by co-HPLC using authentic samples led us to identify the first five fractions as the result of kynurenines and xanthurenic acid derivatives. The 27.3-min fraction had optical absorption bands at 227 and 273 nm and a fluorescence band around 340 nm. Its fast atom bombardment high resolution mass spectrum revealed a molecular mass of 256. FTIR spectra of the sample, measured in KBr pellets, showed absorption bands for the −NH (3,360 cm^{-1}), CO (1,690 cm^{-1}), and aromatic moieties (1,578, 1530 cm^{-1}).

Fig. 2 shows the ^1H and ^{13}C NMR spectra of the compound. The proton NMR spectrum is remarkable in its similarity to that of **III** and of another β-carboline isolated from the root bark of *Perriera madagascariensis* (35), in its aromatic and alicyclic regions, and suggests that the compound under study is another β-carboline. That it has a vinyl moiety was apparent from the signals at 7.32 ppm (dd) and 5.37 ppm (d), and a methyl group was also clear from the 3-proton strong singlet at 1.6 ppm. The assignments of various proton resonances are listed in Table I, which also shows the numbering of the atoms in the molecule. The ^{13}C NMR spectrum of the compound is also shown in Fig. 2 and is quite similar to that of the β-carboline from *P. madagascariensis* which has been identified as 4,7-dimethoxy-1-vinyl-β-carboline (35), although the A-ring heterocycle is not totally conjugated in the present case as is in the latter. The assignment of the resonances to the various carbons is also listed in Table I. On the basis of the NMR assignments, the FTIR, and from an analysis of the fast atom bombardment mass (Fig. 3*A*) and the positive ion electrospray mass spectral profile (Fig. 3*B*) of the molecule shown in Fig. 3, we assign to this compound the structure 1-methyl-1-vinyl-2,3,4-trihydro-β-carboline-3-carboxylic acid and illustrate it as compound **V** in Fig. 1.

Spectral Features of the β-Carbolines—The photophysical and photochemical properties of some β-carbolines have been studied in the literature (11–13). Some of these are phototoxic to bacteria and insects; Larson *et al.* (11) have found the ability to kill test organisms to vary in the order **I** > **II** > **II**a, and **II**b to be inactive. This phototoxicity rank order is not in direct consonance with their ability to produce reactive oxygen species such as $^1O_2$, O_2^-, and H_2O_2 upon irradiation; β-carbolines with a partially saturated pyridine ring (A ring) such as **II**a and **IIb** were found to be more efficient generators of reactive

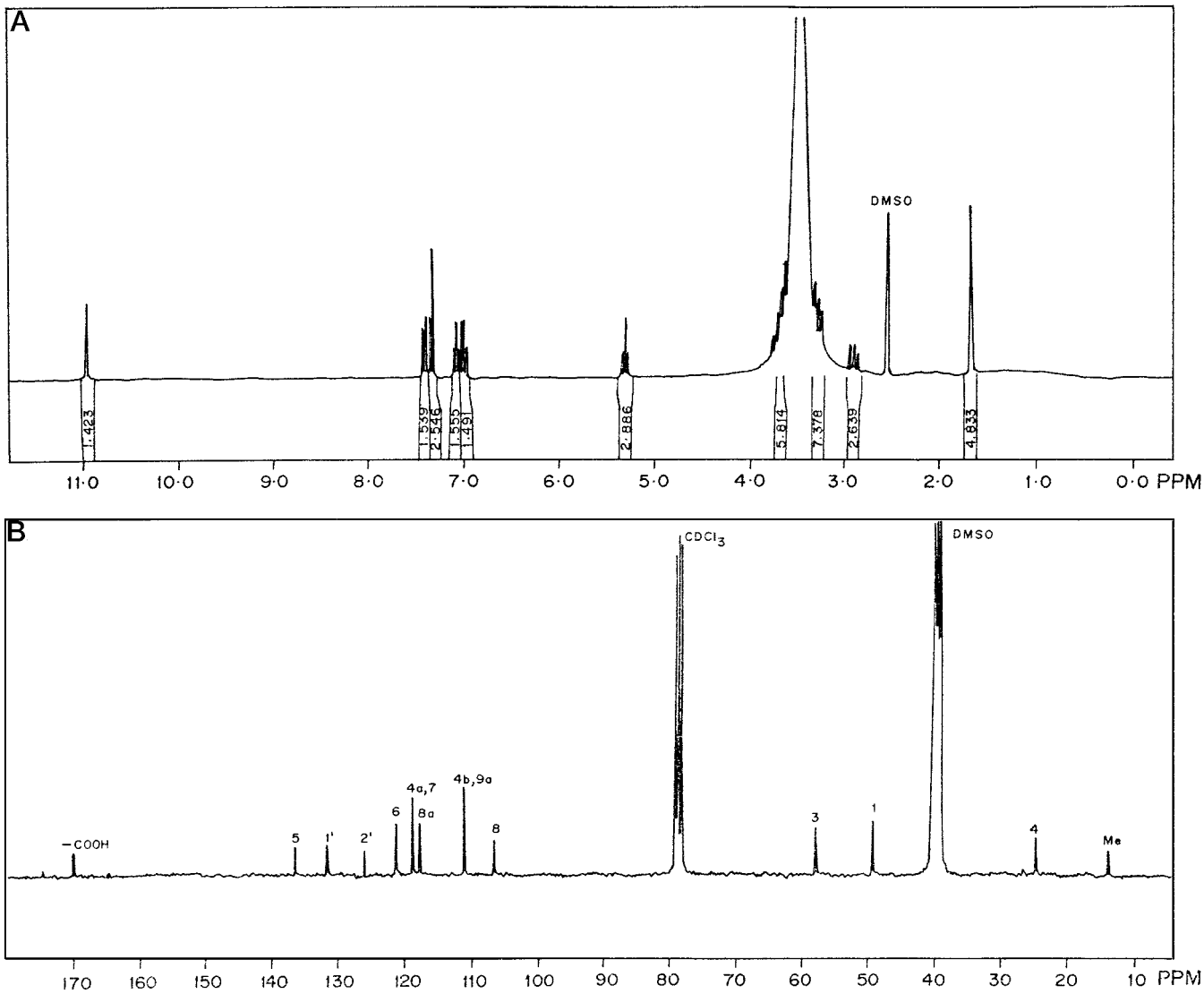

FIG. 2. *Panel A*, ^{1}H NMR spectrum of compound **V**. *Panel B*, ^{13}C NMR spectrum of compound **V**.

TABLE I
^{13}C and ^{1}H NMR spectral data of **V**

Data were recorded in 1:1 $CDCl_3$:dimethyl sulfoxide-d_6 at 300 MHz (for ^{1}H) and 75 MHz (for ^{13}C). s, singlet; d, doublet; dd, doublet of doublet.

Position	$\delta\ ^{13}C$	$\delta\ ^{1}H$
1	49.16	
3	57.66	3.72 (dd, 12.0, 4.5)
4	24.9	2.88 (dd, 12.0, 4.5)
		3.20 (dd, 12.0, 4.5)
4a	118.7	
4b	111.0	
5	136.3	7.42 (d, 7.0)
6	121.2	7.06 (dd, 7.0, 7.0)
7	118.7	7.00 (dd, 7.0, 7.0)
8	106.5	7.31 (d, 7.0)
8a	117.7	
9a	111.0	
1′	131.5	7.32 (dd, 16.0, 9.0)
2′	125.9	5.32 (d, 18.0)
COOH	169.7	
CH_3	13.7	1.6 (s)
9-NH		10.95 (s)

oxygen species than the fully aromatized ones (**I** and **II**), and yet they do little or no photodamage to target cells.

The difficulty encountered in reconciling these two properties might lie in the fact that in a protic environment, particularly water, the fully aromatized compounds can exist in multiple states in equilibrium, namely neutral, cationic, and tautomeric both in the ground and excited states (36); the principal absorbing tautomer may be quite different from the principal emitting or photoactive state. Also, in molecules with reduced conjugation such as **II**a, energy level calculations and fluorescence measurements (value of the parameter $\tau_o = \tau/\phi_F$) indicate that the order of electronic energy levels changes, making the $^{1}(n\pi^*)$ state lower than the $^{1}(\pi\pi^*)$ state, although the former transition is not observed since it is forbidden. Given this background, it appears difficult to predict *a priori* the photodynamic properties of β-carbolines based on their electronic structures and energy levels alone. Instead, Kawashima *et al.* (37) have attempted to address the question by synthesizing a large number of closely related molecules and comparing their pro-oxidant and antioxidant behavior, using the structure-activity correlation approach. Thus, it seemed desirable for us to study the photodynamic properties of each of our β-carbolines experimentally.

We find that all the three compounds, **III**, **IV**, and **V**, show their principal absorption band around 273 nm and fluorescence emission maximum around 348 nm, similar to L-Trp itself, typical of the indole chromophore. The emission quantum yields of these were measured to be 0.27 for **III**, 0.19 for **IV**, and 0.17 for **V**, and their fluorescence life times to be 4.0 ns

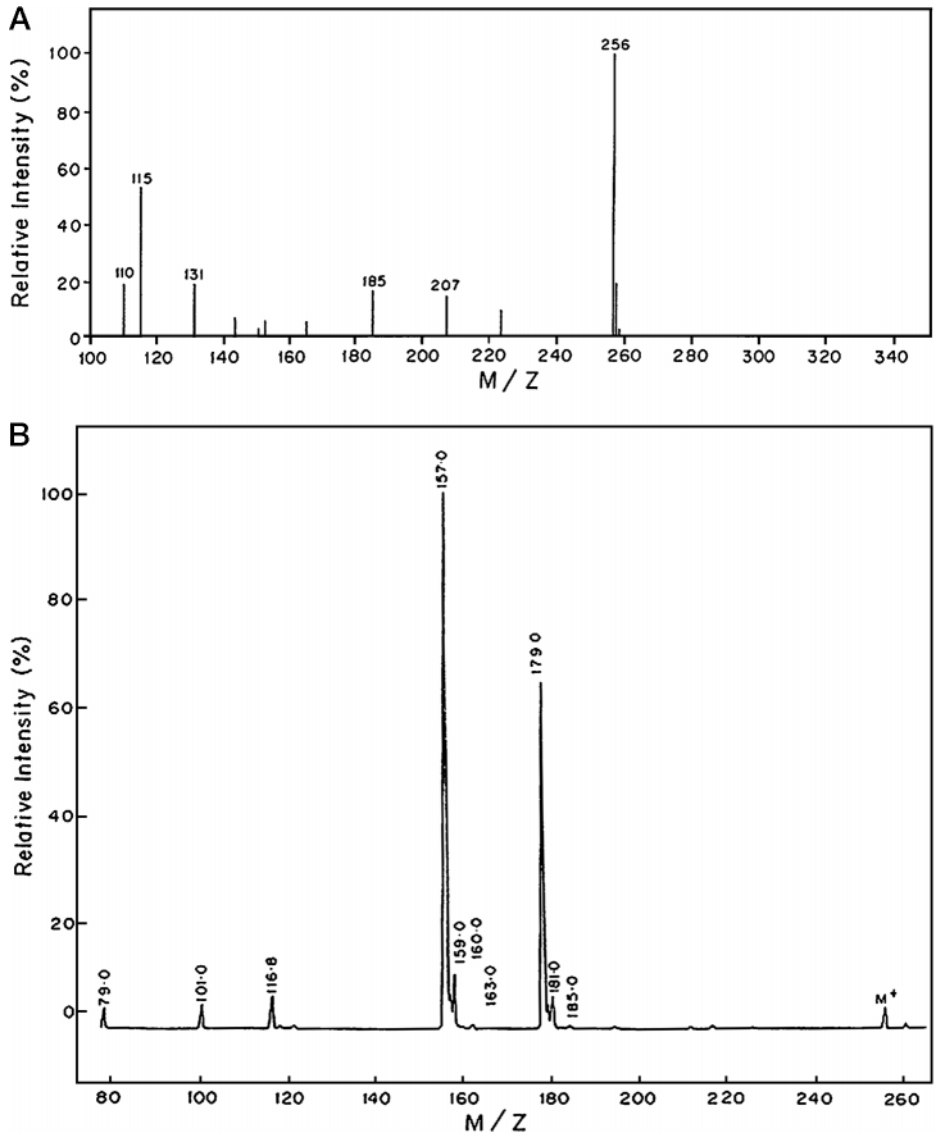

FIG. 3. *Panel A*, fast atom bombardment mass spectrum of compound **V**. *Panel B*, positive ion electrospray ionization mass spectroscopy of compound **V**.

for **III**, 3.1 ns for **IV**, and 4.8 ns for **V**. Based on these, we estimate the τ_o values as 15 ns for **III**, 16.3 ns for **IV**, and 26 ns in the case of compound **V**. (These are larger than the values of 9 and 7 ns, estimated for **I** and **II** (36), and suggest that in these cases too, the lowest state may be $^1(n\pi^*)$. Semiempirical molecular orbital calculations, using the Dewar approach (34), support this possibility though, as expected, the $n\pi^*$ transitions are not seen.)

Photodynamic Properties—Because these properties are of relevance to the β-carbolines that accumulate in tissues such as the skin and the eye, which receive light and might be subject to photic and oxidative stress, we undertook a comparative study of the photodynamic properties of compounds **I–V.** Fig. 4*A* shows the results of the RNO bleaching assay (26), which monitors the ability of these compounds to produce singlet oxygen upon irradiation with light at their absorption frequencies. Whereas harmane and harmine are photodynamic and generate $^1O_2$, the three lenticular β-carbolines are inert in this regard. Fig. 4*B* shows the results of the Fridovich assay (27) in photodynamically generating superoxide radicals. Here too, the lenticular β-carbolines show no sensitizer property, whereas harmane is active on this count; our results on harmane agree with those of Chae and Ham (12). Harmine appears inefficient in producing $O_2^{\cdot-}$, although it is able to generate $^1O_2$.

Each of the β-carbolines, namely **III**, **IV**, and **V**, is thus seen to be neither a pigment that extends the spectral range of the lens nor a pro-oxidant that imposes photodynamic stress on the tissue. Are they then just benign accumulants or do they have any active modulatory role in the tissue? In light of the suspicion that some β-carbolines might possess antioxidant properties (14–16), we investigated this aspect in some detail.

Antioxidant Abilities of the Lenticular β-Carbolines—An effective, rapid, and high-throughput method to evaluate the antioxidant character of various substances has been introduced recently by Rice-Evans and co-workers (29, 30) and is called the ABTS assay. Fig. 5 shows the results of the ABTS assay of the five β-carbolines and compares them with some other compounds that accumulate as Trp metabolites in the body, particularly in the eye. The pro-oxidants quinaldic acid and kynurenic acid enhance the reaction, as expected, whereas kynurenine is inert, not affecting the reaction in any major manner, compared with the control. Neither does harmane or harmine. However, the three lenticular β-carbolines are efficient in their antioxidant capacity, comparable in their ability to the water-soluble vitamin E analog Trolox. It would thus appear that they might function as endogenous antioxidants in the lens and other tissues where they accumulate.

One direct way of testing their antioxidant or oxyradical scavenging ability is to assess their effectiveness in inhibiting

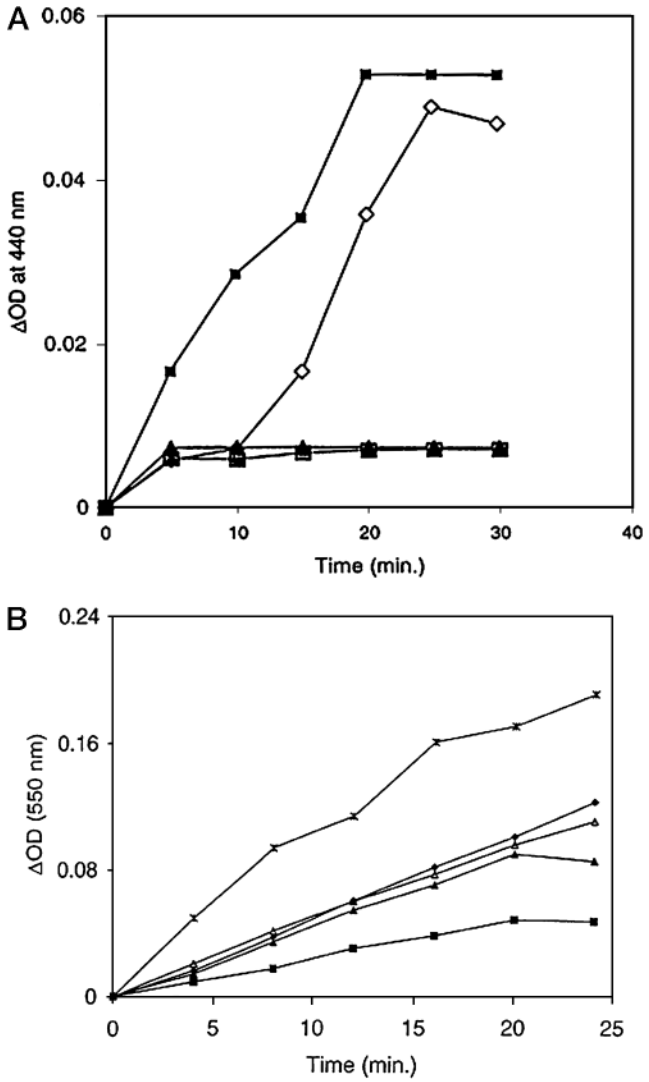

FIG. 4. *Panel A*, photosensitized RNO bleaching measured at 440 nm in the presence of imidazole (10 mM) in 50 mM phosphate buffer, pH 7.4, with 0.1 mM **I** (■), 0.1 mM **II** (◇), 0.1 mM **III** (▲), and 0.1 mM **IV** (□). *Panel B*, photosensitized superoxide production measured as the rate of cytochrome *c* reduction, in the absence (△) and presence of 0.1 mM **I** (*), 0.1 mM **II** (♦), 0.1 mM **III** (▲), and 0.1 mM **IV** (■).

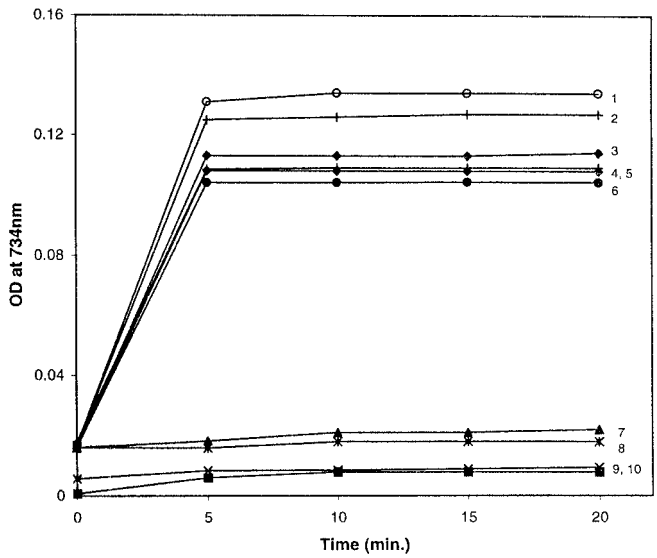

FIG. 5. **Inhibition of ABTS oxidation monitored at 734 nm with metmyoglobin (50 μM) and H_2O_2 (1 mM) in 50 mM phosphate buffer, pH 7.4, in the absence and presence of 250 μM concentrations of various additives.** Listed from *top* to *bottom* are: *1*, quinaldic acid; *2*, kynurenic acid; *3*, compound **I**; *4*, compound **II**; *5*, control (no additives); *6*, kynurenine; *7*, compound **IV**; *8*, Trolox; *9*, compound **III**; and *10*, compound **V**.

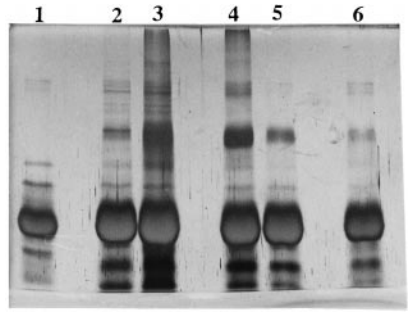

FIG. 6. **Silver-stained SDS-polyacrylamide gel electrophoresis profile of γ-crystallin incubated for 72 h at 37 °C with Fenton reagent ($FeCl_3$ + ascorbic acid).** *Lane 1*, control γ-crystallin (untreated); *lane 2*, γ-crystallin incubated at 37 °C; *lane 3*, γ-crystallin incubated at 37 °C with Fenton reagent; *lanes 4*, *5*, and *6*, γ-crystallin incubated at 37 °C with Fenton reagent in the presence of 1 mM **I,** 1 mM **II,** and 0.5 mM **III**, respectively.

the oxidative covalent cross-linking of proteins which occurs through the Fenton reaction that produces $OH^{\cdot}$. Fig. 6 shows such an experiment on the $OH^{\cdot}$ radical-mediated covalent cross-linking of the test protein γ-crystallin to produce high molecular weight aggregates. Whereas harmane does not inhibit the cross-linking at all, harmine does so mildly, and **III** is quite effective as an inhibitor of the oxidative cross-linking. Compound **IV** was found in a separate experiment (results not shown) to be just as effective as **III**, at comparable molarities. Compound **V** could not be tried because of paucity of sample.

In a comparison experiment, we tried the radical scavenging ability of these compounds by monitoring their effectiveness in inhibiting the photodynamic oxidative covalent cross-linking of γ-crystallin in the presence of the sensitizer riboflavin and irradiation at 445 nm (20). Essentially the same results were obtained; whereas **I** and **II** did not inhibit the formation of high molecular weight species, **III** and **IV** effectively prevented the reaction.

Inhibition of the Direct Photoaggregation of a Lens Protein—The fact that compounds **III**, **IV**, and **V** occur in the human lens led us to the following experiment. The protein γ-crystallin, which is known to be abundant in the core or nuclear region of the mammalian eye lens, is photolabile and precipitates out of solution upon irradiation with light in the 280–300 nm region (31). Fig. 7 shows that this photoaggregation of γ-crystallin is prevented effectively by **III** and **IV** but not by **I** and **II** (compound **V** could not be tried for want of enough material). Although its mechanism needs to be worked out in detail, light-induced aggregation of γ-crystallin is suspected to be radical-mediated because quenchers such as dithiothreitol or histidine suppress the reaction (31). In light of the antioxidant and radical-scavenging properties of the β-carbolines described above, the differential behavior of **I** and **II** on one hand and of **III** and **IV** on the other is as expected.

This differential behavior of these compounds appears to be related to their structural differences. Of the five, harmane and harmine are totally conjugated in their rings (the A-ring is pyridinyl in character, making the system totally aromatized). Their ability to donate hydrogen atoms or electrons is expected to be weaker than those of **III**, **IV**, or **V**, which are saturated (piperidinyl in their A-ring); the latter compounds also carry carboxylic moieties. It is likely that either or both of these features offer these β-carbolines their radical scavenging and antioxidant properties. In this context it is worth noting that

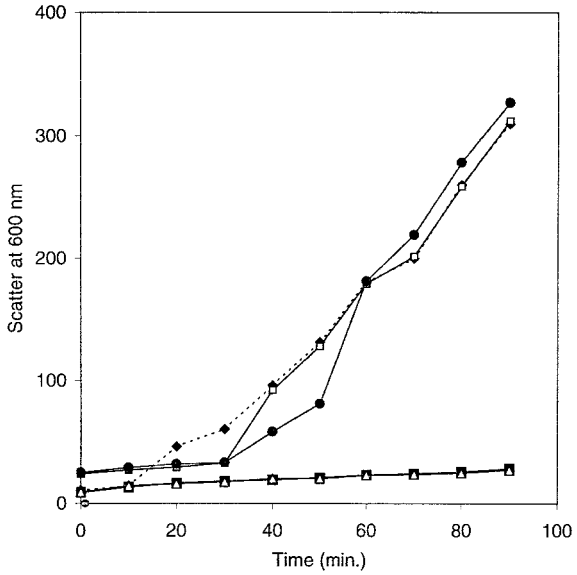

FIG. 7. **Photoaggregation of γ-crystallin.** The protein solution (1 mg/ml) was irradiated at 295 nm in the spectrofluorometer with a 20 nm bandpass; the light intensity was 0. 2 mW/cm^2, 10^{14} photons/s. Light scatter at 600 nm was monitored every 10 min with excitation and emission bandpasses kept at 5 and 3 nm, respectively. Control γ-crystallin (□) and protein in the presence of 1 mM each **I** (●), **II** (♦), **III** (■), and **IV** (△) are shown.

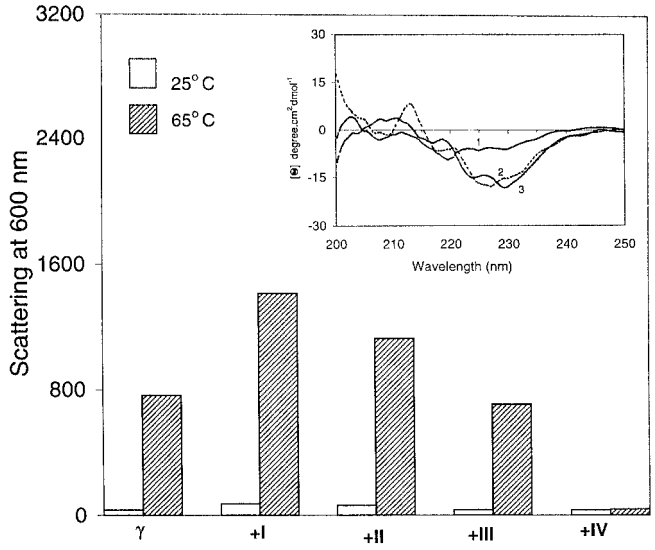

FIG. 8. **Thermal aggregation of γ-crystallin.** Aggregation was monitored at 600 nm at 25 and 65 °C. The *inset* shows the far UV CD spectra of γ-crystallin with 0.5 mM **IV** at 25 °C (*curve 1*), 55 °C (*curve 2*), and 65 °C (*curve 3*).

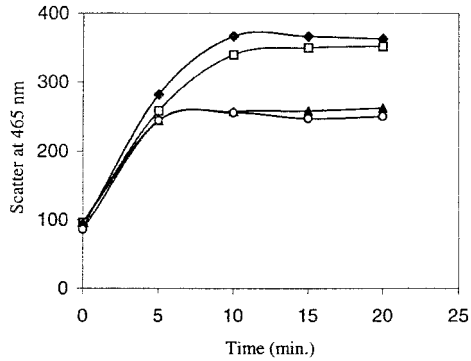

FIG. 9. **Aggregation of insulin B chain.** Aggregation was monitored by measuring the scattering at 465 nm. Shown are 0.2 mg/ml insulin alone (♦) and the protein in the presence of 0.3 mM (□), 1.2 mM (▲), and 2 mM (○) **IV**.

another tetrahydro-β-carboline (1-(3,5-dimethoxyphenyl)-2-propyl-1,2,3,4-tetrahydro-β-carboline) has been found to be a potent inhibitor of lipid peroxidation and cyanide intoxication in mice (37). It is thus possible that the saturated or alicyclic feature of the A-ring in β-carbolines provides the antioxidant character. It is relevant to point out that the ABTS assay follows the electron or hydrogen-donating ability of reducing agents to reduce the ferryl myoglobin back to metmyoglobin; we wonder whether the differential behavior of **I** and **II** in their ability to reduce ABTS (Fig. 5) reflects structural differences (the 1,2 double bond in **I** is reduced in **II**, providing two extra hydrogens). Kawashima *et al.* (37) have noted that the electronic charge and the electron-withdrawing character at position 1 are important for the antioxidative property.

Solubilizing Ability of **IV** toward Proteins—γ-Crystallin also precipitates upon heating (32), and the reason is thought to be simply a thermal conformational alteration rather than any redox process. One would thus expect no effect of the β-carbolines on the heat-induced light scattering of γ-crystallin. We followed the effect of heating solutions of γ-crystallin to 65 °C in the absence and presence of 1 mM β-carbolines by measuring the increase in light scattering at 600 nm. Fig. 8 shows a surprising result; whereas **I**, **II**, and **III** do not affect the thermal coagulation of γ-crystallin (and even enhance it a little), the β-carboline diacid **IV** effectively prevents this aggregation. When we estimated the minimum concentration of **IV** needed to prevent such thermal aggregation of γ-crystallin, we obtained the mole ratio **IV**:protein = 250:1. It is likely that the dicarboxylate binds to the protein through coulombic and/or hydrophobic forces and inhibits protein-protein interactions that lead to phase separation. Interestingly, this is in the range of physiological concentrations. In an effort to check whether the interaction is apolar in nature, we followed the emission spectral features (wavelength maximum and intensity) of **IV** in the presence of micelles of the cationic surfactant cetyltrimethylammonium bromide and of the neutral surfactant Triton X-114. Although the fluorescence of 0.1 mM **IV** was affected (blue shifted from 348 to 338 nm and intensity dropped by about 20%) in 10 mM cetyltrimethylammonium bromide, the neutral detergent TX-114 did not have any effect. This leads us to suggest that the interaction between **IV** and γ-crystallin might also be predominantly electrostatic in nature.

The fact that γ-crystallin remains in solution even upon heating up to 65 °C in the presence of **IV** has enabled us to monitor its conformation. γ-Crystallin denatures upon heating beyond 72 °C (38) and thus should have much of its native structure intact at 65 °C. The *inset* in Fig. 8 shows that the secondary structure of γ-crystallin is largely unaltered in the presence of **IV** upon heating up to 65 °C. Thus the thermal coagulation of the protein is largely a tertiary structural effect. We could not access the tertiary structural features of the protein in this instance because the high amounts of **IV** present in the solution (1 mM) interfered with both the fluorescence spectrum of the protein and its near UV CD spectrum.

However, this solubilizing ability of **IV** is not general or as effective in all cases, and we have found mixed results. Fig. 9 shows that increasing amounts of **IV** are able to inhibit the nonthermal aggregation of insulin. When insulin is reduced upon the addition of the disulfide reagent dithiothreitol, the reduced molecules interact and entangle, leading to increased light scattering and eventual precipitation. Proteins such as α-crystallin, which act in a chaperone-like fashion, are able to inhibit this aggregation and keep the insulin molecules in solution (39, 40). When we tried **IV** as the solubilizing agent,

we found it to be just about 60% as effective as α-crystallin; and with other aggregating proteins such as α-lactalbumin and β-crystallin, **IV** was not able to prevent their precipitation and light scattering. This leads us to suggest that the solubilizing ability of β-carboline is more a reflection of complexation and electrostatic binding with individual proteins than of the generalized mechanism of providing a hydrophobic surface to which the unfolded or aggregation-prone target protein can bind (32, 40). It is also to be added that **IV** has so far been seen in the lens only in the protein-bound form and not as free molecules for its action. Even in the free form, one would require millimolar amounts of the substance to effect its solubilizing action. It may thus serve as a potential solubilizing agent with choice proteins *in vitro*.

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