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310 mμ ABSORBANCE IN *PHYSALIA PHYSALIS*: DISTRIBUTION OF THE ABSORBANCE AND ISOLATION OF A 310 mμ ABSORBING COMPOUND

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Abstract—1. The Portuguese man-of-war, *Physalia physalis*, contains extractable material which absorbs intensely at 310 mμ.

2. The highest 310 mμ absorbance per unit dry weight is found in portions of the float and in the ampullae that synthesize the nematocysts.

3. A pigment, with $\lambda_{max} = 310$ mμ ($\epsilon \sim 5 \times 10^5$) shifting to 298 mμ in strong acid, has been purified from *Physalia* float autolysate.

4. It contains β-alanine, β-aminoisobutyric acid and probably tryptophan or a derivative thereof.

5. It has a molecular weight of about 1200.

INTRODUCTION

IN A PUBLICATION on carbon monoxide synthesis in the gas gland of *Physalia physalis*, Wittenberg (1960) noted the presence of strong u.v. absorbance at 260 and 305 mμ in the water extract of the gas gland. The very high intensity of the absorption in the 305 mμ range is in itself of interest, since very few known physiological compounds have an absorbance maximum in the vicinity of 310 mμ.

In this paper we report the results of work undertaken to determine the anatomical distribution of the 310 mμ absorbing substance(s) in *Physalia*, and to begin characterization of the 310 mμ absorbing compound(s).

METHODS AND RESULTS

Distribution of 310 *mμ absorbance*

A fresh specimen of *P. physalis* with a 28-cm long float was placed on a glass plate over a white background and dissected into eighteen portions representing different morphological components described in the footnote to Table 1. After each portion was thoroughly homogenized in distilled water, it was placed in a centrifuge tube, diluted with 1 vol. of ethanol, the whole mixed thoroughly and then allowed to stand for at least 5 min before being centrifuged at 27,000 *g* for 10 min. The pellet was extracted a second time by thoroughly dispersing in 67% ethanol

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and resuspending several times before the suspension was centrifuged at 27,000 *g* for 15 min. Following a third extraction with 1% ammonia, each pellet was dried at 100°C, desiccated and weighed.

The 310 mμ absorbance of each of the fifty-four solutions (except for first extracts Nos. 7 and 8 in which the 310 mμ absorbance was only a shoulder and had to be estimated graphically) was determined as the difference between the absorbance at 370 mμ and that at 310 mμ. The $A = 0$ point was chosen as 370 mμ since $A_{370} = 0$ for the purified 310 mμ absorbing compound (Fig. 3). The volume of each solution having non-zero A_{310} was measured before the three extracts of each of the eighteen portions were combined and dried for determination of the weight of non-volatile material.

The results of these assays are listed in Table 1, and the anatomical sections from the *Physalia* specimen in the footnote to Table 1.

310 *mμ Absorbance in nematocysts*

Nematocysts were isolated according to the method of Lane & Dodge (1958) with some modifications: (1) tentacles were not washed free of sand upon collection, (2) the tentacles were frozen for 3 weeks before they were allowed to autolyze for 8 days at 4°C, (3) the autolyzed tentacles were screened through a 200-mesh screen to remove virtually all the sand present and (4) the homogenization of the nematocysts was carried out in synthetic sea water rather than in distilled water.

Ultraviolet spectra were recorded from the diluted autolyate solution, all the washes, the homogenate suspension and the supernatant after centrifugation of the homogenate. The clean nematocysts were checked microscopically to assure that they were, in large proportion, undischarged. The pellet from the nematocyst homogenate was similarly checked to be sure that virtually all the nematocysts had been fragmented.

The diluted autolysate of about 30 g of tentacles, decanted from the settled nematocysts, contained a large amount of 310 mμ absorbance, about $1500A_{310}$ (ml). (For definition, see note with Table 1.) The cloudy nematocyst homogenate contained no 310 mμ absorbance detectable above background absorbance. The centrifuged homogenate showed no 310 mμ absorbance.

Isolation of a 310 *mμ absorbing compound*

About 200 *Physalia* floats were collected, deflated by cutting and allowed to autolyze for 2 days in sea water at ambient temperature (*ca.* 23°C). The absorption spectrum of this solution at a 1 : 10 dilution in distilled water is shown in Fig. 1. After unautolyzed material was removed from *ca.* 20 l. of solution by filtration, about 50 g of Darco G-60 charcoal was added to it to remove materials easily adsorbed at pH 7. The autolysate was siphoned off the settled charcoal and made 1% in acetic acid before treatment with about 250 g of Darco G-60 charcoal to adsorb the 310 mμ material present. After the charcoal had settled, the autolysate solution was siphoned off and discarded. The charcoal slurry was made 50% in ethanol and neutralized for temporary storage. At pH 7 the 310 mμ absorbing

material is not adsorbed on the charcoal, so the charcoal was removed from the ethanolic solution by filtration before the solution was made into a methanolic solution by repeated evaporation to low volume and dilution with methanol. Upon addition of methanol to the water–ethanol solution, a white precipitate, containing no 310 mμ absorbing material, formed. The evaporation and addition of methanol were repeated until no further precipitation occurred. The resulting crude methanolic solution was stored at −12°C until used.

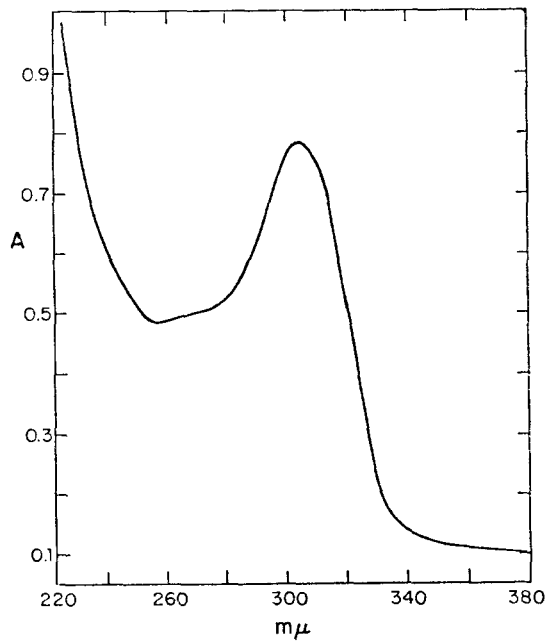

FIG. 1. Absorption spectrum of a crude autolysate, appropriately diluted, from *P. physalis*.

Crude extract was evaporated to a syrup, taken up in elution buffer (0·2 M citrate, pH 2·7), and acidified to pH 2·5 with formic acid before it was put on a Dowex 50W-X2 cation-exchange-resin column (68 × 1·5 cm) equilibrated with elution buffer. The elution pattern from the column is shown in Fig. 2. The third 310 mμ absorbing fraction eluted from the column contained most of the absorbance. This fraction was titrated to pH 8 with ammonium hydroxide before it was evaporated to small volume and applied to a Dowex 1-X2, formate form, column (26 × 1·5 cm). The column was eluted first with water, then with 0·02 M ammonium formate, pH 8. The single 310 mμ absorbing fraction eluted from the column was evaporated to small volume, the concentrated solution placed on a Sephadex G-10 column (75 × 1·5 cm) and the column eluted with deionized water. A single zone of 310 mμ material was recovered from this column. This solution

was then evaporated to low volume and applied to paper (Whatman No. 3 MM) which was subjected to electrophoresis at pH 3·6 for 85 min at 44 V/cm in a solvent of 5% acetic acid, 1% pyridine. A single homogeneous zone of 310 mμ absorbing material which had moved 6·2 cm toward the anode was identified on the paper. It was removed from the paper by elution with water. The material so obtained will be referred to henceforward as *H* substance. When subjected to electrophoresis

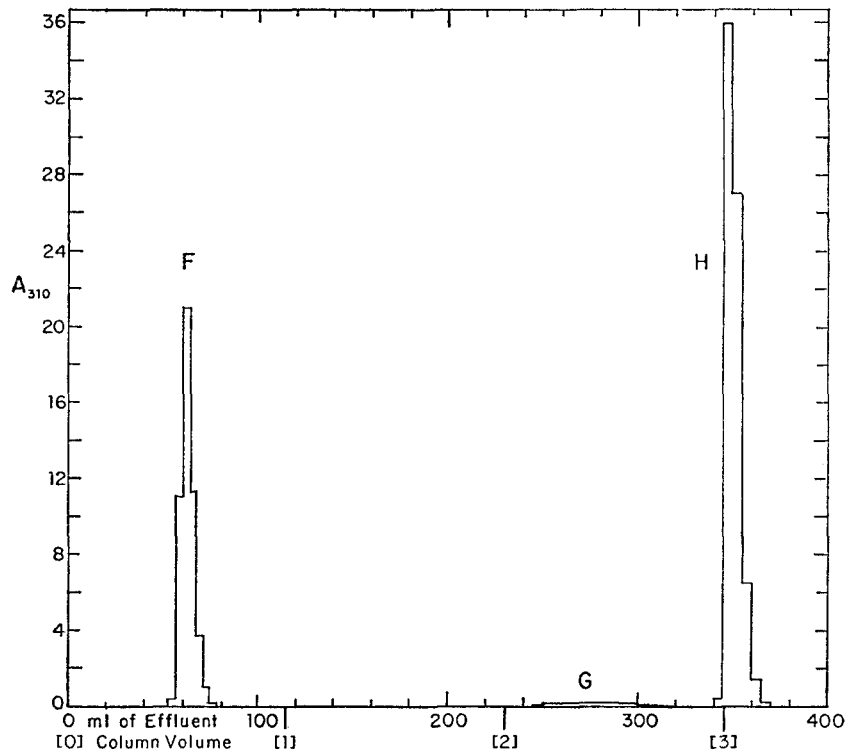

FIG. 2. Elution pattern (assayed by 310 mμ absorption) from an AG 50W-X2 ion-exchange column of partially purified extract from *Physalia*.

(at a variety of pH values), thin-layer chromatography (silica as absorbent, methanol as solvent) and gel filtration (see later), this material appeared to be a single homogeneous species. It gives a positive reaction with ninhydrin, and this has been used to some extent to follow the purification. However, at least in the earlier stages, the amount of contaminating ninhydrin-reacting material is so large that the diagnostic value for *H* substance is limited.

Characterization of H substance

Absorption spectra. From pH 4 to 13 no significant change occurs in the spectrum. At pH 0·1 the absorption maximum shifts to 298 mμ. Since no

isosbestic point occurs in this change, the shift is not caused by a single simple protonation.

Using the equation: $pKa' = \text{pH} + \log (A \text{ buffer} - A \text{ NaOH})/(A \text{ acid} - A \text{ buffer})$ to determine the pKa' of the group causing the spectral shift, a value of 1·4 is obtained for the left side of the peak, and 1·8 for the right side. This confirms that the spectral shift is not due to a single protonation.

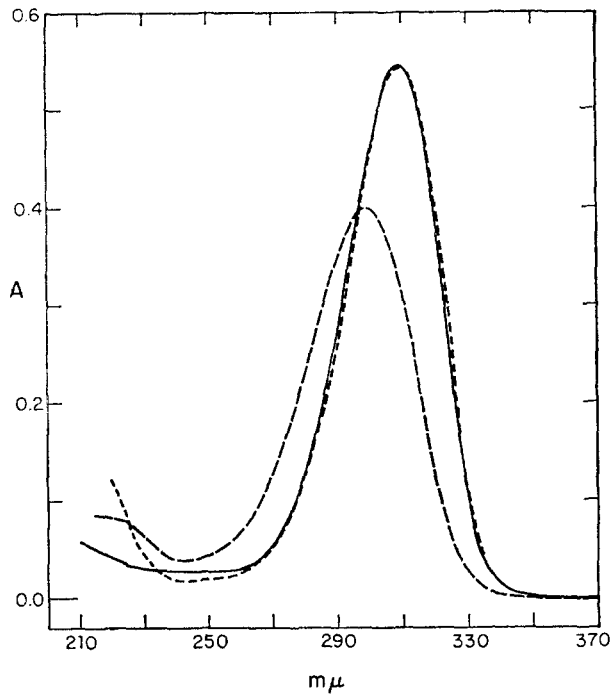

FIG. 3. Absorption spectra of *H* substance. ———, pH 7; - - - - -, pH 13; – – – –, pH 0·1.

Since the molecular weight of *H* substance is not known, the molar absorptivity cannot be determined directly; however, since molar amounts of known amino acids can be determined after acid hydrolysis of solutions with known absorbance values, an expression can be derived for the molar absorptivity in terms of the number of such moieties per molecule. The molar absorptivity, ϵ, equals $A/(b \times c)$, where A is the measured absorbance, b is the length (cm) of the optical path and c is the molar concentration of the compound. If ϵ' is defined as $\epsilon' = A/(b \times c')$, where c' is the molar concentration of a moiety which appears n times in the compound, then $c = c'/n$ and $\epsilon = n\epsilon'$. Sixty-one A_{310} (ml) units yielded 0·087 μmoles of β-aminoisobutyric acid. ϵ' is therefore 6×10^5. In view of this high absorptivity, it seems likely that $n = 1$ and ϵ also equals 6×10^5.

Fluorescence spectra. Fluorescence spectra of *H* substance were recorded with a Mark I spectrofluorometer (Farrand Optical Company). A 284–340 mμ excitation–emission pair is the principal feature of the spectrum.

Isoelectric point. The isoelectric point of *H* substance was determined by finding the pH at which the material did not move from the zero point in high-voltage paper electrophoresis at different pH values. Movement of the material

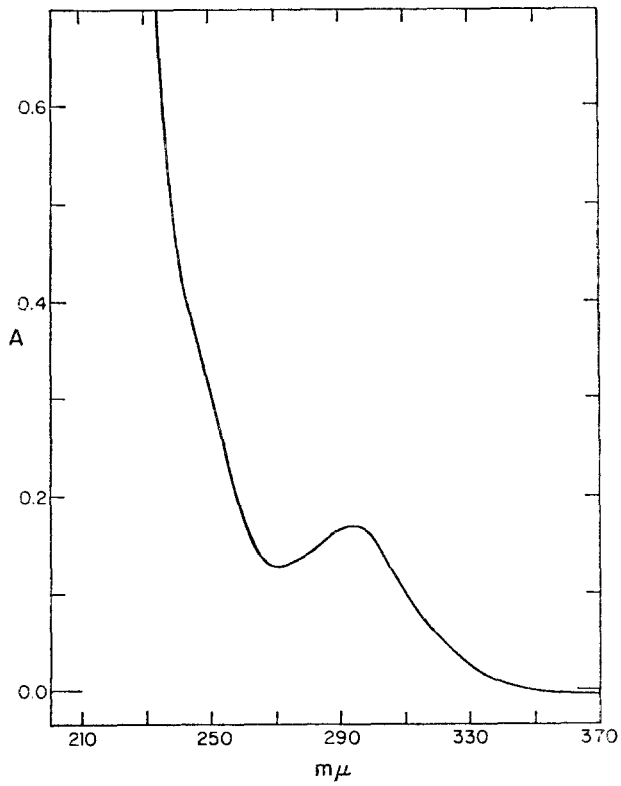

FIG. 4. Absorption spectrum of *H* substance after 19 hr at room temperature, pH 14.

was judged by ninhydrin reaction, by u.v. absorption on the dried paper (viewing it with a 260 or 360 mμ lamp), and by the presence of 310 mμ absorption in an aqueous extract of pieces cut from the electrophoresis paper.

The value so obtained was pH $2{\cdot}6 \pm 0{\cdot}3$.

Molecular weight. Two attempts were made to determine the molecular weight of *H* substance on the analytical ultracentrifuge (Beckman Model E). In the first the solvent was aqueous potassium chloride (1 N); in the second it was methanol. In both attempts a cell layering solvent over the sample solution was used. *H* substance diffused to the meniscus in each run, and it failed to sediment after 3 hr at 292,000 *g*.

A second technique used was gel filtration. On Biogel P-2 in distilled water the sample volume to void volume ratio (V_s/V_0) of *H* material was 1·15. Since absorption may occur (although other experiments in buffer solutions indicate that it does not), this datum, which corresponds to a molecular weight on the order of 1300, can be taken to imply only a minimum value.

Lastly, a Diaflo UM-2 filter, which has a molecular weight cut-off in the vicinity of 1000, retarded *H* material so that the filtrate is approximately one-third as concentrated as the solution retained. This indicates that the molecular weight of *H* material is not considerably higher than 1000.

Thus the molecular weight of *H* material appears to be in the range 1000–1500.

Constituent amino acids. *H* substance was subjected to acid hydrolysis with 6 N hydrochloric acid in an evacuated sealed tube for 22 hr at 110°C. After removal of the hydrochloric acid in a desiccator over sodium hydroxide, the hydrolysate was taken up in pH 2·2 buffer and run on a Beckman amino-acid analyzer. The molar amounts of amino acids were computed by the standard procedure. Acid hydrolysis of $61A_{310}$ (ml) of *H* substance yielded 0·493 μmoles of β-alanine and 0·087 μmoles of β-amino isobutyric acid. No other amino acids were detected.

In an attempt to determine whether tryptophan is present in *H* substance, perhaps in modified form, a sample was refluxed in ethanol over Raney nickel for 2 days (to cleave a sulfur–indole bond such as is present in *Amanita* peptides). The sample was washed off the nickel with 1% ammonium hydroxide and the solution was evaporated to dryness; the residue was subjected to basic hydrolysis in an evacuated sealed tube with sodium hydroxide (10 N) plus a crystal of stannous chloride for 5 hr at 110°C. The hydrolysate was adsorbed on Dowex 50W-X2 (H^+-form) and the column eluted with 1·0 N ammonium hydroxide. The ammoniacal eluate was evaporated to dryness and the residue, taken up in the standard buffer (pH 2·2), was run on the amino-acid analyzer. Tryptophan was tentatively identified on the basis of the elution pattern from the column.

Darkening of acid hydrolysate. The acid hydrolysates of *H* material all contained considerable dark, charred material, while the hydrolysates of paper blanks remained clear. Such darkening can be produced when either tryptophan or carbohydrate is present in the material.

DISCUSSION

At least three distinct molecular species, with similar absorption spectra, are present in the concentrated methanolic extract from an autolysate of the float of *P. physalis*. Two of these may well be artefacts produced by autolysis or in the isolation procedure. Thus we have studied the one (*H* substance) which appears to have the largest molecular weight (gel filtration) and to be the most abundant (50 per cent of the 310 mμ absorbance in the extract).

From Table 1, the amount of 310 mμ absorbance in the float (2070 units) can be compared with the amount in the rest of an individual *Physalia* (9120 units). Despite this greater preponderance in the pendant polyps, the highest concentrations per unit dry weight is in the float, and this is of interest in view of the

TABLE 1

Tissue homogenate No.	A_{310} (ml)*	Percentage of total A_{310} (ml)*	Dry wt. (g)	$\frac{A_{310}\text{ (ml)*}}{\text{Dry wt. of tissue homogenate}} \times 10^{-2}$
1	850	7·59	1·0228	8·3
2	921	8·23	0·7406	12·4
3	13	0·12	0·0168	7·7
4	65	0·58	0·0953	6·8
5	96	0·86	0·1170	8·2
6	69	0·62	0·0717	9·6
7	11	0·10	0·1596	0·7
8	16	0·14	0·1349	1·2
9	6919	61·82	10·4	6·7
10	160	1·43	0·2203	7·3
11	42	0·38	0·0506	8·3
12	108	0·96	0·1728	6·2
13	404	3·61	0·1750	23·1
14	32	0·29	0·0248	12·9
15	278	2·48	0·3658	7·6
16	76	0·68	0·0937	8·1
17	642	5·74	0·5008	12·8
18	490	4·38	0·3036	16·1
Total (whole organism)	11,200	99·74	14·67	

Material included in each tissue homogenate

(a) Pendant polyps
1. That part of each of three large fishing tentacles distal to the ampulla.
2. Three ampullae with attached parallel sections of fishing tentacle.
3. One tubular attachment of a large fishing tentacle to the float severed at the codon and at the proximal end of the ampulla.
4. Small, unpigmented or lightly pigmented fishing tentacles including the sections apparently beginning to develop into ampullae.
5. Intermediate size, pigmented fishing tentacles severed below the ampullae.
6. Small ampullae from the type of fishing tentacles included in No. 5 above.
7. Gonozoids and other polyps on the same branch.
8. Gastrozoids.
9. All the remaining appendages less the short stalks immediately adjacent to the float.
10. The short, unbranched stalks immediately adjacent to the float.

(b) Floats
11. The gas gland with a small amount of the surrounding saccus (pneumatosaccus).
12. The rest of the saccus except for a very small portion cut off with the apical pore.
13. The sail of the codon (pneumatocodon).
14. The apical pore tip of the codon plus a very small amount of the attached saccus.
15. The bulge section of the codon to which the pendant polyps are attached.
16. The oral tip of the codon.
17. The remainder of the oral half of the codon after removal of Nos. 13, 15 and 16 above.
18. The remainder of the apical pore end of the codon after the removal of the crest and a pical pore tip.

Note: Descriptions of *P. physalis* anatomy may be found in articles by Totton (1960), Mackie (1960) and Lane (1960).

* In determining the distribution of 310 mμ absorbance and in the isolation procedures it was necessary to express the amount of 310 mμ absorbing material present. An arbitrary unit of 310 mμ absorbance, an A_{310} (ml), read "*A*-310 millilitre", was defined as the amount of material that would give rise to $A_{310} = 1$ when dissolved in 1 ml of water at pH 7 and read in a cell with a 1-cm path length.

speculations presented below. Only the ampullae of the large fishing tentacles have equivalent concentrations. Nematocysts, on the other hand, have practically no 310 mμ absorbance.

Since the expected V_s/V_0 ratio for a molecule of mol. wt. = 1000 on Biogel P-2 is 1·3, it can be inferred that *H* substance with a $V_s/V_0 = 1{\cdot}15$ has a molecular weight somewhat above 1000.

The isoelectric point of *H* substance is low for a compound from which only neutral amino acids have been isolated after hydrolysis. At present we have no explanation for the acidic nature of the compound.

Concerning these amino acids, only two have been identified positively, and these are rather unexpected. They are β-alanine and β-aminoisobutyric acid. They occur in a 5 : 1 ratio. Tryptophan has been tentatively identified, but the molar ratios of these are not known. A 5 : 1 : 1 ratio of these three amino acids would give a molecular weight of 644 (assuming amide linkage), approximately half the value derived from gel-filtration experiments. Doubling the numbers in this ratio would, however, increase the value for the molar absorptivity (which is based on one β-aminoisobutyric acid residue). It seems very reasonable that the remainder of the molecule contains as yet undetected residues, possibly carbohydrate in nature (see the unusual amount of browning occurring in the acid hydrolysis).

Since neither of the amino acids identified after acid hydrolysis has a significant fluorescence spectrum, the observed 284–340 mμ excitation–emission pair may be assigned to the presence of typtophan (which would be destroyed on acid hydrolysis), but it could arise from another indole-containing residue, or from some quite different chemical structure.

The u.v. absorption spectrum of the purified *H* substance has a number of unusual features. The peak is very sharp—at half-height it extends from 290 to 335 mμ—and the absorbance at 240–250 mμ is very low (3 per cent of the absorbance at 310 mμ). Its molar absorptivity, calculated on the basis of amino acids liberated on acid hydrolysis from a solution of known absorbance, is $\epsilon = n \times (6 \times 10^5)$, where n = number of β-aminoisobutyric acid residues per molecule. It seems likely that $n = 1$. Even if there are several chromophores per molecule, this absorptivity is exceptionally high (compare carotenoids with $\epsilon \sim 10^4$–10^5).

Since the total units of 310 mμ absorbance in the float, divided by its approximate horizontal cross-sectional area (in cm^2), is 10, almost total absorption of light of that wavelength, and between the range of 290–335 mμ, seems likely. Thus although the habitat and nature of *Physalia* make it liable to very high exposure to visible and u.v. irradiation, the organism is shaded from the 290–335 mμ band, which in mammalian skin, for example, is largely responsible for sunburn (Daniels *et al.*, 1968). The negative charge which the compounds bear at neutral pH could be involved in binding it to specific structures intracellularly or in the outer surface of the organism (its location in this respect has not yet been determined).

Other possible functions for the 310 mμ absorbing compound, e.g. as an energy trap or transducer or as a precursor-molecule for a compound with physiological activity, produced, then, in response to light, are feasible but there is no evidence

for them. On the other hand, the very high absorptivity and the remarkably high concentration of the compound in *Physalia* lend support to the idea that the absorption is functional. Further work in this area remains to be done.

Finally, some interesting parallels should be pointed out at this time between *H* fraction and the cyclic peptides, described and characterized by Wieland (1964), from the *Amanita* species of mushrooms. A typical member of this group of toxic peptides, α-amanatin, has, for example, a similar absorption maximum, although its absorbance increases markedly below 260 mμ as compared with *H* substance. Both compounds contain unusual amino acids although *H* substance seems to have many fewer and different amino acids. The chromophores in the two compounds may well be very similar or identical on the basis of our tentative evidence for tryptophan in *H* substance. The intriguing question then on pharmacological activity for *H* substance (although it should be emphasized that it does not occur as such in nematocysts), either through its resemblance to the *Amanita* peptides or by virtue of its content of β-alanine and β-aminoisobutyric acid (both neuron-blocking agents), remains to be answered.

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Key Word Index—Ultraviolet damage, protection from; protection from u.v. damage; *Physalia physalis*; β-alanine; β-aminoisobutyric acid; chromophore, u.v. absorbing; pigment, u.v. absorbing; gas gland of *P. physalis*; nematocysts of *P. physalis*; sunburn, protection from; *Amanita* peptides, chromophore in; radiation, protection from u.v.; energy sink, u.v. radiation; u.v. pigment; Portuguese man-of-war.