β-3-Oxindolylalanine (Hydroxytryptophan)

2. SPECTROSCOPIC AND CHROMATOGRAPHIC PROPERTIES

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The synthesis of β-oxindolylalanine from isatin and ethyl pyruvate is described in the preceding paper (Cornforth, Cornforth, Dalgliesh & Neuberger, 1951). During the course of this work the chromatographic behaviour of an apparently pure specimen of the amino-acid on filter paper was examined; it was found in the earlier preparations that the synthetic substance gave, apart from the main spot, a weak subsidiary spot indicating the presence of a small amount of material with hydrophilic characteristics. This finding led to a more extensive investigation covering other compounds metabolically related to tryptophan or oxindolylalanine.

The second part of this paper is concerned with the ultraviolet absorption of oxindolylalanine and various other substituted oxindoles and the effects of changes of pH and oxidation on the absorption spectra. A comparison of the behaviour of 3-methyloxindole and that of the new amino-acid confirmed the structure assigned to the latter on the basis of the synthesis. Finally, the structure of phalloidin is discussed on the basis of its reported absorption spectrum as compared with that of oxindolylalanine.

Chromatography

EXPERIMENTAL

General. Paper chromatography was carried out in the usual way using Whatman no. 4 paper. The compounds were applied as 0·2% solutions with the exception of kynurenic acid which was an approximately saturated solution containing considerably less than 0·2%.

Colour reactions used on paper. With ninhydrin, oxindolylalanine and kynurenine gave purple colours, whilst with tryptophan a greyish-blue colour was produced. Kynurenine, but none of the other three compounds, gave a red colour on diazotization with nitrous fumes and subsequent coupling with alkaline β-naphthol. On spraying with 2% (w/v) *p*-dimethylaminobenzaldehyde in 5% (w/v) HCl (Ehrlich's reagent) kynurenine gave an orange colour immediately, oxindolylalanine a pale-yellow colour and tryptophan a violet colour that developed much more slowly. Millon's reagent gave with tryptophan a pale-yellow colour immediately on spraying, whilst with oxindolylalanine an orange spot developed more slowly. On drying at about 70° the latter became deep orange and the tryptophan spot became pale orange.

It was shown by Wieland, H. & Witkop (1940) that oxindolylalanine responds to the colour reaction of Folin & Denis (1912) for phenols; this test has now been applied to paper chromatograms. The phosphomolybdic-tungstic acid solution was sprayed on the previously dried chromatogram, the paper was allowed to dry and then sprayed with 2N-Na_2CO_3. Oxindolylalanine gave a blue colour and tryptophan a pale-green colour, the colours sometimes appearing before the carbonate spraying. It was found important to use an all-glass sprayer when using the Folin-Denis reagent. With kynurenine no colour was produced.

Fluorescence on illumination with ultraviolet light. Kynurenic acid did not give a colour with any of the reagents tried, but it was clearly visible when illuminated with ultraviolet light. The light source used was an 'Osira' lamp (General Electric Company Ltd.) fitted with a Wood's glass envelope; the lamp emitted mainly between 3000 and 3500 A. Kynurenic acid on paper gave a blue-green fluorescence and kynurenine a strong pale-blue fluorescence. Tryptophan and oxindolylalanine did not fluoresce when acidic solvents were used for irrigation. However, when alkaline solvent mixtures had been used, spots fluorescing weakly on ultraviolet illumination appeared with both amino-acids. These spots did not coincide with the spots revealed on subsequent spraying with ninhydrin and are considered to be due to decomposition products formed during the prolonged exposure to alkaline solvents.

RESULTS

R_F values of oxindolylalanine, tryptophan, kynurenine and kynurenic acid

Many solvent systems were examined and the results obtained with some of these are shown in Table 1. Owing to the instability under alkaline conditions of most of the compounds investigated, basic solvent systems are not to be recommended; moreover, they offer no advantage with respect to resolution. In acidic systems kynurenic acid appeared to be faster than tryptophan, oxindolylalanine and kynurenine, which had similar R_F values with all the usual solvent mixtures examined. Tryptophan, kynurenine and kynurenic acid could readily be separated, especially when some of the more specific colour tests described above were applied; but none of the usual solvent systems was found capable of resolving all the four substances. Anomalous retardation on development of the filter-paper chromatograms with water (Synge & Tiselius, 1949) was shown by all three amino-acids, but the R_F values were again very similar.

Table 1. *R_F values of tryptophan, oxindolylalanine, kynurenine and kynurenic acid found with different solvent systems*

(In all but one experiment, the ascending technique of Williams & Kirby (1948) was used. The descending technique of Consden, Gordon & Martin (1944) was employed in the experiment using phenol-NH_3. Where figures are given in parentheses, the solvent was allowed to run off the paper and the figures represent the distances (in cm.) travelled.)

Solvent mixture		R_F value			
Composition	Reference	Tryptophan	β-Oxindolyl-alanine	Kynurenine	Kynurenic acid
n-Butanol-acetic acid-water (4:1:5)	Partridge (1946)	0·54	0·52	0·50	0·66
Phenol containing 0·3% NH_3 (w/v) and a trace of NaCN	Consden *et al.* (1944)	(19·2)	(17·9)	(16·9)	—
Collidine-2:4-lutidine-water (1:1:2)	Dent (1948)	0·79	0·84	0·86	0·00
n-Butanol-benzyl alcohol saturated with water (1:1)	—	0·225	0·235	0·22	—
n-Butanol-propionic acid-water (50:25:35)	Calvin & Benson (1949)	0·56	0·53	0·50	—
n-Butanol-propionic acid-water (4:1:5)	—	0·45	0·41	0·36	—
n-Butanol-formic acid-water (4:1:5)	—	0·65	0·60	0·57	—
Amyl alcohol-pyridine-water (35:35:30)	de Verdier & Ågren (1948)	0·48	0·42	0·38	0·41
Water	Synge & Tiselius (1949)	0·68	0·75	0·78	0·74

Resolution and differentiation of tryptophan, oxindolylalanine and kynurenine was readily obtained when formaldehyde was incorporated into an acidic solvent mixture. Thus, with a homogeneous mixture formed from *n*-butanol, glacial acetic acid, 40% (w/v) formaldehyde solution and water (5:1:1:3 by vol.), kynurenine on subsequent treatment with ninhydrin gave a brown spot with R_F 0·77, oxindolylalanine gave a purple spot with R_F 0·63 and tryptophan gave two yellow spots with R_F values 0·68 and 0·93 respectively. In a mixture these spots were separate and could be readily distinguished. The slow-moving tryptophan spot corresponded to that given by 2:3:4:5-tetrahydro-β-carboline-4-carboxylic acid under the same conditions.

Chromatographic investigation of the purity of synthetic oxindolylalanine

Early preparations of oxindolylalanine which had been recrystallized and gave correct elementary analyses, gave on paper chromatography with various solvent mixtures two spots. The major spot had R_F values (reported in Table 1) close to those of tryptophan, whilst the minor spot corresponded to a more hydrophilic substance with R_F values which ranged with the acidic solvent mixtures between 0·09 and 0·28. In later preparations the second slower-moving spot was not observed.

This minor constituent of the early preparations reacted on paper with ninhydrin but not with the reagents of Millon, Ehrlich or Folin & Denis, and to obtain further information about its nature an attempt was made to isolate it on a column of paper pulp using the procedure of Campbell, Work & Mellanby (1951). From 1·5 g. of crude oxindolylalanine there was thus obtained 15 mg. of material, which did not melt below 350°, but charred. Analysis of this material (found: C, 53·0; H, 6·7; N, 5·8%) was not inconsistent with its being a tetrahydroquinoline derivative, but in view of the small amount available its exact nature was not established. In any case, the amount obtained from the chromatographic separation shows that it did not constitute more than 1–2% of even the earliest preparation.

Absorption Spectra

EXPERIMENTAL

All melting points are uncorrected.

Preparation of compounds. The preparation of β-oxindolylalanine and of 1-benzyloxindole is described by Cornforth *et al.* (1951). Oxindole was purified by repeated crystallization from water followed by sublimation *in vacuo*; it had m.p. 126°. 3-Methyloxindole was prepared by cyclization of β-propionylphenylhydrazine (Brunner, 1896, 1897); after steam distillation, recrystallization from water and sublimation *in vacuo*, it had m.p. 124°. 3:3-Dimethyloxindole was prepared from β-*iso*butyrylphenylhydrazine (Brunner, 1897) and purified in a similar manner; it had m.p. 152°. 1:3:3-Trimethyloxindole was prepared from α-methyl-β-*iso*butyrylphenylhydrazine (Brunner, 1897).

The product after distillation at 140–145°/16 mm. was converted into the $HgCl_2$ complex (Julian, Pikl & Boggess, 1934) which was crystallized successively from ether and aqueous ethanol; it had m.p. 125°; Julian *et al.* give 122·5° for the m.p. of the $HgCl_2$ complex. On decomposition with 2N-NaOH, extraction with ether and removal of the solvent, 1:3:3-trimethyloxindole was obtained as a colourless oil, b.p. 98–99°/1 mm.

Dioxindole was prepared by reduction of isatin with $Na_2S_2O_4$ (Marschalk, 1912) and was purified by repeated crystallization from benzene; it had m.p. 166–168°.

Measurements of absorption spectra. Most of the compounds were dissolved in water to give 10^{-3}–10^{-4}M-solutions. 1:3:3-Trimethyloxindole and 1-benzyloxindole were first dissolved in methanol; water or NaOH was then added to give a final concentration of 20% (v/v) methanol. The absorption spectra were measured as soon as possible after making up the solutions. Any solution not under observation was kept stoppered and in the dark. No special precautions were taken to exclude air. In the experiment in which the rate of change of molecular extinction coefficient of 3-methyloxindole at 340 mμ. with time was measured, the material was dissolved in water and an equal volume of 0·2N-NaOH added at zero time. A similar technique was employed with other compounds in measuring the immediate changes of absorption on addition of alkali.

The measurements were made with the Beckman spectrophotometer Model DU, employing 1 cm. cells and a hydrogen lamp.

RESULTS

Absorption spectra in neutral and acid solution

The absorption spectra of oxindole, 1-methyloxindole and 1:3:3-trimethyloxindole have been measured by Ramart-Lucas & Biquard (1935) who found that the spectra of these three compounds were similar, all having a maximum ($\log \epsilon = 4{\cdot}0$) at 250mμ. All oxindole derivatives examined by us had maxima in neutral or acid solutions in positions which varied between 248 and 252 mμ. (Table 2); the ε values ranged from 7250 to 9000. At short wavelengths there was a minimum at 225 mμ. with ε ranging between 3000 and 3500. At longer wavelengths absorption fell sharply (Figs. 1–3), but all oxindole derivatives examined had a 'shoulder' between 270 and 285 mμ. with ε values falling from 2250 to 1000. This 'shoulder' was somewhat less marked in acid solution, but otherwise the spectra were almost identical at pH 1 and 7. The spectra of oxindolylalanine, its α-*N*-acetyl derivative (Fig. 6) and 3-methyloxindole were very similar in neutral solution (Figs. 1 and 6).

Changes of absorption spectra in alkaline solution

Immediate changes. All oxindole derivatives possessing an imino-hydrogen atom such as oxindole, 3-methyloxindole and 3:3-dimethyloxindole showed on addition of alkali an immediate shift of the absorption maximum amounting to about 8 mμ. This instantaneous change was followed in the case of oxindole and of 3-methyloxindole by a much more marked, but progressive alteration of the spectrum. The immediate shift of the maximum on addition of alkali did not occur with the two derivatives not possessing an imino-hydrogen atom, namely 1:3:3-trimethyloxindole and 1-benzyloxindole. However, with the latter, there were observed progressive changes similar to those found with other oxindole derivatives, containing one or two hydrogen atoms in the 3-position. The immediate shift which appeared on addition of alkali was seen most clearly with 3:3-dimethyloxindole (Fig. 2), where it was not obscured by secondary changes. Fig. 3 shows that with 1:3:3-trimethyloxindole the spectra in acid and alkaline solution were identical.

Progressive changes. When oxindole was left in 0·1N-sodium hydroxide for several hours, there was found intense absorption at wavelengths below 245 mμ., a maximum at 256·5 mμ. ($\epsilon = 7950$) and a second peak at about 365 mμ. ($\epsilon = 2400$). This spectrum did not change any further and resembled that of isatin. Similar changes were also found with 1-benzyloxindole.

Addition of alkali to a solution of 3-methyloxindole produced a change in the spectrum extending over about 2 hr., the appearance of a new peak at 340–344 mμ. being most marked. The rate of change, as measured by the increase of extinction at 340 mμ., is shown in Fig. 4. The spectra of oxindolylalanine

Table 2. *Positions of maxima and minima (mμ.) and the corresponding extinction coefficients of various oxindole derivatives*

	Solvent							
	Water				0·1N-HCl			
Substance	$\epsilon_{max.}$	$\lambda_{max.}$	$\epsilon_{min.}$	$\lambda_{min.}$	$\epsilon_{max.}$	$\lambda_{max.}$	$\epsilon_{min.}$	$\lambda_{min.}$
Oxindole	7680	248·5	3450	225·0	8600	248·5	3000	223·5
3-Methyloxindole	—	—	—	—	8000	249·0	3400	226·0
3:3-Dimethyloxindole	7680	248·5	3400	225·0	8090	248·5	3000	223·5
1:3:3-Trimethyloxindole	8950	252·5	—	—	—	—	—	—
1-Benzyloxindole	8470	251·0	3400	227·5	—	—	—	—
β-Oxindolylalanine	7250	250·0	3250	227·5	8450	251·0	3570	227·5
α-*N*-Acetyl-β-oxindolylalanine	—	—	—	—	7300	252·0	3000	224·5
Dioxindole	—	—	—	—	(*a*) 5300	(*a*) 252·0	(*a*) 3800	(*a*) 231·5
					(*b*) 1450	(*b*) 292·0	(*b*) 1100	(*b*) 276·0

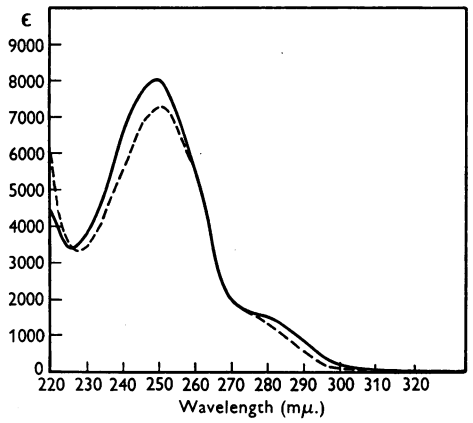


Fig. 1. Absorption spectra of β-oxindolylalanine, – – –, and of 3-methyloxindole, ——, in water.

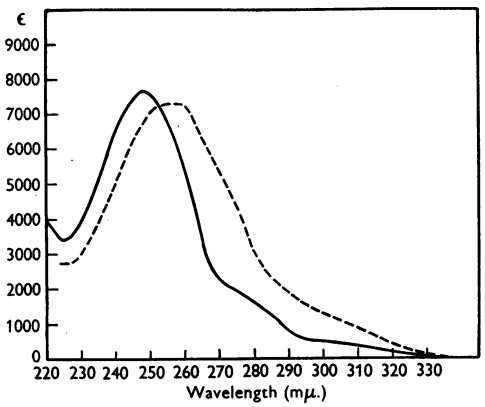


Fig. 2. Absorption spectra of 3:3-dimethyloxindole in water, ——, and in 0·1 N-NaOH, – – –.

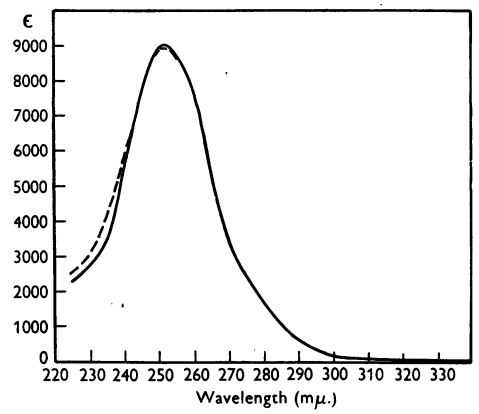


Fig. 3. Absorption spectra of 1:3:3-trimethyloxindole in neutral 20% (v/v) methanol, ——, and in 0·1 N-NaOH containing 20% (v/v) methanol, – – –.

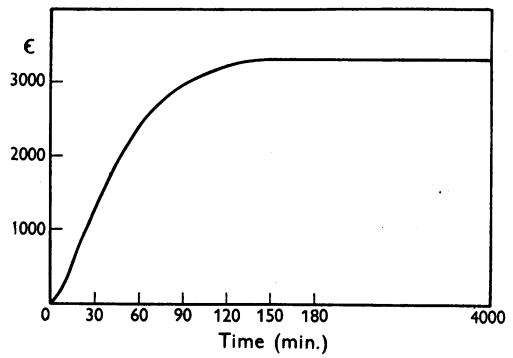


Fig. 4. Rate of increase with time of molecular extinction coefficient at 340 mμ. of 3-methyloxindole in 0·1 N-NaOH.

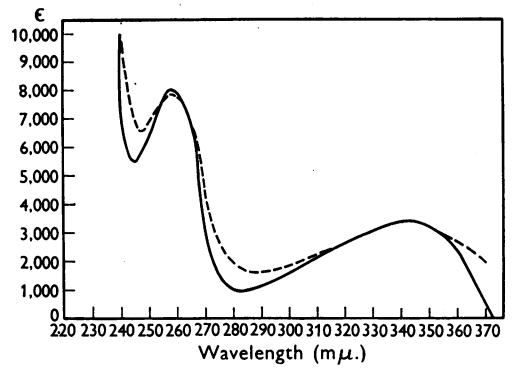


Fig. 5. Absorption spectra of β-oxindolylalanine, – – –, and 3-methyloxindole, ——, in 0·1 N-NaOH. Spectra were measured on solutions which had been left standing at room temperature for 4 hr.

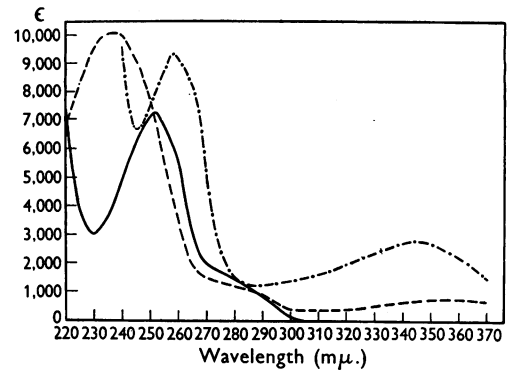


Fig. 6. Absorption spectra of α-*N*-acetyloxindolylalanine in 0·1 N-HCl, ——, in 0·1 N-NaOH, –·–·–), and in 0·1 N-HCl after exposure to 0·1 N-NaOH for 4 hr., – – –.

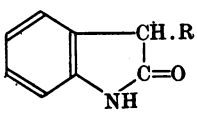
and its acetyl derivative, after exposure to 0·1 N-sodium hydroxide for 4 hr., were almost identical with that of 3-methyloxindole measured under similar conditions (Figs. 5 and 6). All three spectra showed two maxima, one at 340–344 mμ. and the other at about 258 mμ., and in addition intense absorption at wavelengths below 245 mμ.

When a solution of 3-methyloxindole in 0·1 N-sodium hydroxide was left to stand for 4–16 hr. and acidified until the concentration of hydrochloric acid was 0·1 N, the spectrum was indistinguishable, from that of a solution (Fig. 1) that had never been made alkaline at all. But exposure to alkali for longer periods produced irreversible changes, as shown by the shift of the maximum (in acid) from 250 to 240 mμ. and a marked increase of absorption at wavelengths below 240 mμ. With oxindolylalanine and its acetyl derivative the changes brought about by exposure to alkali could not be reversed by acidification. This is shown in Fig. 6 for the acetyl derivative. With the free amino-acid the changes, as observed on the acidified solution, were even more marked. A solution of oxindolylalanine which had been left in 0·1 N-sodium hydroxide for 18 hr. and was then acidified to pH 1·0 showed marked absorption in the near ultraviolet with a maximum at 360 mμ. ($\epsilon = 1600$) and intense absorption below 260 mμ. without an apparent maximum. This irreversible change may be due to oxidation to a dioxindole derivative; but dioxindole itself gives a somewhat different spectrum (Table 2).

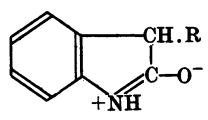
DISCUSSION

The structure of oxindolylalanine

The resemblance between the absorption spectrum of the synthetic amino-acid and those of other oxindole derivatives and particularly the close similarity between the spectrum of oxindolylalanine exposed to alkali for some time and that of similarly treated 3-methyloxindole, leave no possible doubt that the structure assigned to the new compound is correct. As already pointed out by Ramart-Lucas & Biquard (1935) the fact that the spectrum of oxindole is very similar to that of 1-alkyl-3:3-dialkyl- or 1:3:3-trialkyl-oxindoles indicates that oxindoles generally are best represented by formula (I) and the resonance structure (II); the enol form or the lactim form do not appear to be important contributors to the structure of oxindole.



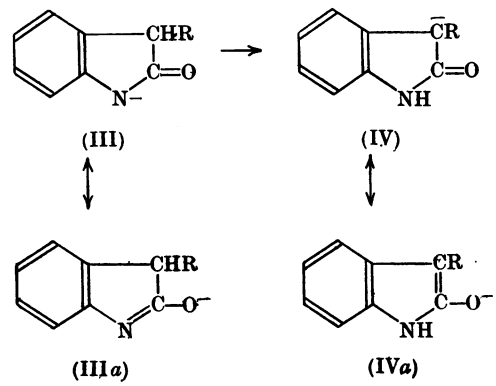
(I)



(II)

Changes on addition of alkali

The primary and instantaneous changes in spectral absorption on addition of alkali appear to depend on the availability of a hydrogen atom in the 1-position and not on the presence of a hydrogen atom in the 3-position. It would thus appear that ionization of oxindole as an acid consists primarily of a removal of the imino-hydrogen as a proton giving (III) and its resonance hybrid (III*a*). On the other hand, the secondary and progressive changes in absorption consisting of the appearance of new bands at 340 or 260 mμ. are associated with the presence of at least one hydrogen atom in the 3-position. Oxindole itself is readily oxidized in alkaline solution to dioxindole and isatin, and the spectral changes observed in this case are almost certainly associated with such an oxidation. With 3-methyloxindole the slow spectral changes on addition of alkali are at least at first reversible. These are best explained by a prototropic change (III → IV), similar to that found in ordinary enolization.



(III) (IV)

(III*a*) (IV*a*)

The enolate (IV) or (IV*a*) appears to be readily oxidized and this probably explains the irreversible changes found with the 3-alkyloxindoles.

The purity of oxindolylalanine

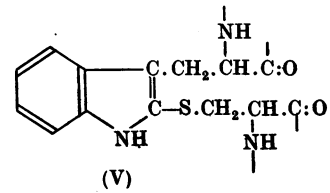
Both the spectrophotometric and chromatographic experiments have shown that oxindolylalanine is unstable in solution, particularly in an alkaline medium. The presence of a secondary spot in the paper chromatograms of the earlier preparations of the new amino-acid may be due to the presence of an impurity in the crystalline material, but the chromatographic, analytical and spectrophotometric results suggest that the amounts of such a contaminant is very small.

The structure of phalloidin

The data on the ultraviolet absorption of oxindolylalanine reported here have a bearing on the constitution of phalloidin. This toxic substance from

Amanita phalloides is a cyclic polypeptide and yields on hydrolysis alanine, cysteine, *allo*hydroxyproline and oxindolylalanine (Wieland & Witkop, 1940). Of the four amino-acids isolated, only oxindolylalanine absorbs light to any extent above 250 mμ. Two absorption curves of this polypeptide have been published; the first by Wieland, H. & Hallermayer (1941), who report two maxima in close proximity at 257 and 251 mμ. with log ε values of 4·31 and 4·35 respectively, a minimum at about 275 mμ. and a further maximum at 290 mμ. (log ε = 4·0). Wieland, T. (1949) reports a maximum at 290 mμ. (log ε = 4·1), a minimum at 257 (log ε = 3·5) and intense absorption at shorter wavelengths. The discrepancy of the two curves in the region below 275 mμ. is not easy to explain, but both papers report a maximum at 290 mμ. with a high extinction coefficient. The similarity of the spectra of oxindolylalanine and its acetyl derivative suggests that incorporation of this amino-acid into a peptide will not affect its absorption spectrum greatly. The finding, therefore, that phalloidin has a maximum at 290 mμ. instead of 250 mμ. must mean that either this peptide contains another light-absorbing structure not yet isolated, or that the oxindole ring is modified in phalloidin. Wieland, H. & Witkop (1940) believed that they had accounted for all the constituents of phalloidin and it is unlikely that a compound, which would have to be present in relatively large amounts to account for the high extinction coefficient, has been overlooked. Thus, it is probable that the oxindole ring is not present in phalloidin, but arises by hydrolysis of the peptide.

Wieland, H. & Witkop (1940) also reported that, provided oxygen is excluded during the hydrolysis of phalloidin, a positive nitroprusside reaction is obtained, indicating the presence of cysteine. On the other hand, phalloidin itself does not yield a precipitate of lead sulphide on treatment with alkaline plumbite (Lynen & Wieland, U., 1937). It thus seems that the thiol group like the oxindole group is masked in the peptide itself. It may therefore be suggested that phalloidin contains the structure (V) which might be expected to give an absorption spectrum of the same type as indole.



(V)

This type of structure on hydrolysis with acid might yield oxindolylalanine and cysteine.

SUMMARY

1. The R_F values of β-oxindolylalanine, tryptophan and kynurenine were measured employing various solvent mixtures. With the usual systems resolution was poor; however, separation was obtained with a butanol-acetic acid mixture containing formaldehyde.

2. The ultraviolet absorption spectra of a number of oxindole derivatives including β-oxindolylalanine have been measured. Addition of alkali was found to produce various reversible and irreversible changes which were correlated with the mode of substitution.

3. A comparison of the reported absorption spectra of phalloidin with those of oxindole derivatives indicates that the oxindole structure is not present as such in phalloidin.

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