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## Spectrophotometric and chromatographic determination of tryptophan dipeptides

In the course of a recent investigation on the radiolysis of tryptophan-containing dipeptides it became necessary to devise a suitable analytical method for measurement of their radiolytic loss. The procedure used has been described briefly[1]. Further details are presented here.

The method is based on the condensation of tryptophan with *p*-dimethylaminobenzaldehyde (DAB), which has been investigated by SPIES AND CHAMBERS[2,3]. It is not specific for tryptophan but for the indole nucleus, and various indole-containing compounds will give different extinction coefficients (see ref. 2 and references quoted therein). Since on radiolysis of tryptophyl dipeptides a mixture of indolic compounds could be expected, it was necessary to separate these from the original dipeptide before measurement of its loss. Thin-layer chromatography proved to be excellent for this purpose, and at the end of this paper some chromatographic results for both the original dipeptides and some of their radiolysis products are given.

Extinction coefficients for the tryptophyl dipeptides were measured during an investigation of the DAB assay with solid, dissolved, or adsorbed dipeptide. The effect of reaction time for the condensation on optical density was also investigated.

*Materials*

Sources of the tryptophyl dipeptides and their possible radiolysis products have been given elsewhere[1]; *p*-dimethylaminobenzaldehyde (DAB), sodium nitrite, and the chromatographic solvents were products of Fisher Chemical Co. All compounds were used as supplied. Precoated thin-layer sheets of cellulose and silica gel were obtained from Eastman ("Chromagram" sheets) and run in the Eastman chromatography tank. A Beckman DB spectrophotometer was used throughout.

*Procedures and results*

As a dipeptide more nearly approximates free tryptophan than it does a protein, some of the procedures described by SPIES AND CHAMBERS[2] for tryptophan alone were tested. Using tryptophyl-tyrosine the following procedures based on those of SPIES labelled E and G (ref. 2) were applied without variation of the temperature, wavelength or sodium nitrite concentration, but with the reaction time for the condensation reduced for convenience from 24 to 18 h. The results are shown in Fig. 1, line A.

*Procedure I.* To a known weight of the solid dipeptide was added 30 mg of solid DAB. 10 ml of 19 *N* sulphuric acid were then added, the flask covered with Parafilm, and agitated until the solids had dissolved. After 18 h in the dark 0.1 ml of 0.04 % soldium nitrite solution was added; the solutions were then shaken, kept in the dark for 30 min, and the optical density read at 590 nm.

*Procedure II.* To 1 ml of an aqueous solution of the dipeptide were added 30 mg of solid DAB and 9 ml of 19 *N* sulphuric acid. Subsequent treatment was as in procedure I.

*Procedure III.* An aliquot of solution was deposited on part of a thin-layer sheet, which was then dried. The portion of the layer containing the dipeptide was

lifted from the sheet, transferred to a small flask and treated in a similar manner to the solid in procedure I. Both cellulose and silica gel layers were tested but only the former was found to be satisfactory.

All three of the above procedures give the same result for the extinction coefficient, indicating that they could be used interchangeably.

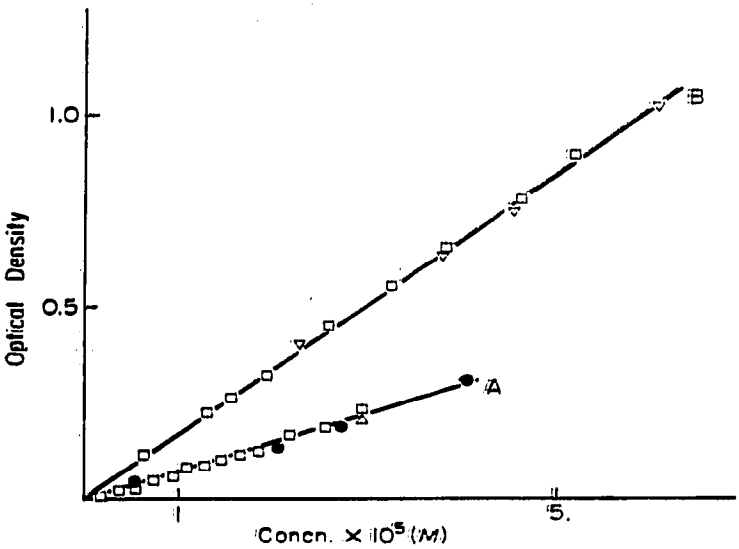


Fig. 1. Calibration graphs for *p*-dimethylaminobenzaldehyde complexes of tryptophyl-tyrosine (A) and tryptophyl-tryptophan (B). ●, Procedure I; □, procedure II; △, procedure III (reaction time 18 h in each case); and ▽, procedure II (reaction time 72 h).

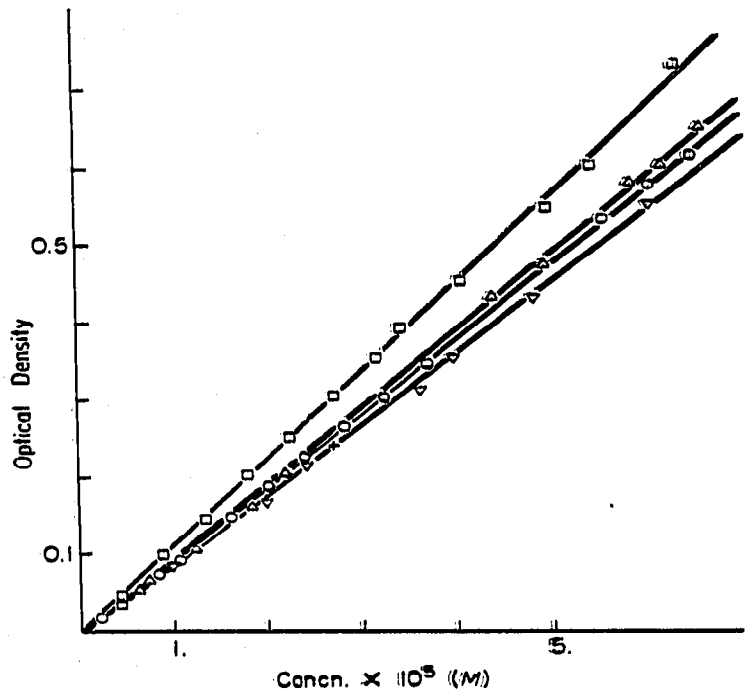


Fig. 2. Calibration graphs for *p*-dimethylaminobenzaldehyde complexes of tryptophyl-phenyl-alanine (△), tryptophyl-glycine (○), tryptophyl-leucine (□), tryptophyl-alanine (▽). Procedure II in all cases except tryptophyl-alanine (+), in which case procedure III was applied.

The effect of reaction time for the condensation reaction on optical density is shown in Fig. 1, line B, for tryptophyl-tryptophan. Using procedure II, the solutions were allowed to stand for 72 h instead of 18 h before the addition of the sodium nitrite. Again no variation in the optical density was observed. It was thus established that the analysis could be carried out on the dipeptides in any form (solid, solution, or adsorbed), and with any time between 18 and 72 h for the condensation reaction, without effect on the result. Procedure II was then repeated with the tryptophyl dipeptides of alanine, phenylalanine, glycine and leucine with the results shown in Fig. 2. Extinction coefficients were calculated from the slopes of these lines and have been reported[1].

Having established the interchangeability of the procedures, attempts were made to quantitatively recover dipeptide from a chromatogram. Aliquots of aqueous solutions of the dipeptides were chromatographed in the appropriate solvent system (generally *n*-butanol–acetic acid–water, 12:3:5) and treated as described in procedure III, after the spots had been located by UV fluorescence. A constant recovery rate of about 90 % was achieved, and the true amount of dipeptide remaining after

TABLE I

$R_F$ VALUES (±0.05) FOR TRYPTOPHAN DIPEPTIDES AND SOME RADIOLYSIS PRODUCTS

Solvent systems: (A) *n*-butanol–acetic acid–water (12:3:5) and (B) methyl ethyl ketone–*n*-butanol–water–acetic acid (90:15:15:2).

| | *Silica gel* | | *Cellulose* | |
|---|---|---|---|---|
| | *A* | *B* | *A* | *B* |
| *Dipeptides* | | | | |
| Tryptophyl-glycine | 0.49 | 0.20 | 0.52 | 0.06 |
| Tryptophyl-alanine | 0.56 | 0.27 | 0.69 | 0.22 |
| Tryptophyl-tyrosine | 0.66 | 0.37 | 0.67 | 0.43 |
| Tryptophyl-tryptophan | 0.75 | — | 0.69 | 0.47 |
| Tryptophyl-leucine | 0.82 | — | 0.72 | 0.48 |
| Tryptophyl-phenylalanine | 0.72 | — | 0.76 | 0.39 |
| *Radiolysis products* | | | | |
| Tyrosine | 0.17 | 0.19 | 0.56 | 0.27 |
| Alanine | 0.21 | 0.03 | 0.29 | 0.07 |
| Leucine | 0.37 | 0.20 | 0.64 | 0.37 |
| Tryptophan | 0.50 | 0.30 | 0.58 | 0.41 |
| Phenylalanine | — | 0.26 | 0.61 | 0.39 |
| Glycine | 0.27 | 0.04 | 0.16 | 0.05 |
| 3,4-Dihydroxyphenylalanine | 0.33 | 0.17 | 0.40 | 0.17 |
| Kynurenine | 0.53 | — | 0.42 | 0.06 |
| 3-Hydroxykynurenine | 0.50 | — | 0.48 | 0.21 |
| Formylkynurenine | 0.56 | — | 0.76 | 0.82 |
| Anthranilic acid | 0.88 | — | — | — |
| 3-Hydroxyanthranilic acid | 0.76 | — | — | — |
| Tryptophanamide | 0.61 | 0.34 | 0.58 | 0.40 |
| Tryptamine | 0.71 | 0.60 | — | 0.60 |
| 3-Indoleacetic acid | 0.79 | 0.91 | 0.96 | 0.97 |
| 3-Indolepyruvic acid | 0.74 | 0.91 | 0.91 | 0.97 |
| 3-Indolecarboxylic acid | — | 0.91 | — | 0.91 |
| Pyruvic acid | — | — | 0.86 | — |
| *p*-Hydroxyphenylpyruvic acid | — | 0.80 | 0.92 | 0.89 |
| Phenylpyruvic acid | — | — | 0.94 | — |
| Tyramine | 0.66 | — | — | — |

radiolysis was calculated by proportionality. Losses in the above process were believed to be due to (a) mechanical losses in transfer and (b) "tailing" of the spots and the non-inclusion of the ""tail"" in the portion of the layer taken for analysis.

The dipeptides, together with a number of possible radiolysis products, were chromatographed under the following standard conditions to provide a method of product identification. Of each compound 0.5 mg, dissolved in a suitable solvent, was applied to the sheet and dried with warm air. The sheet was run in the Eastman tank to a distance of 15 cm, without pre-equilibration of the solvent with the atmosphere. Both cellulose and silica gel were used with the solvent systems *n*-butanol–acetic acid–water (12:3:5) and methyl ethyl ketone–*n*-butanol–water–acetic acid (90:15:15:2). Compounds were detected by spraying with solutions of ninhydrin and/or *p*-dimethylaminobenzaldehyde[4] for amino acids and indoles, and 2,4-dinitrophenylhydrazine[5] for carbonyls. The $R_F$ values obtained are shown in Table I.

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