A New Reagent for the Assay of Indole in the Tryptophanase Reaction

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Harley-Mason & Archer (1958) introduced *p*-dimethylaminocinnamaldehyde as a spray reagent for the detection of indole derivatives on paper chromatograms. This vinyl analogue of *p*-dimethylaminobenzaldehyde gave intensely coloured condensation products with a tenfold increase in sensitivity. The following describes the use of *p*-dimethylaminocinnamaldehyde as a substitute for the benzaldehyde derivative, in a modification of the indole-assay procedure developed by Happold & Hoyle (1934) for the measurement of tryptophanase activity.

EXPERIMENTAL

Indole-extraction procedure. The procedure for extraction of indole into light petroleum, as described originally by Happold & Hoyle (1934), has since been modified in these laboratories. Details of the modified procedure used in the present work are as follows.

Aqueous solutions containing indole are made to a total of 20 ml. Most of the indole is then extracted into 40 ml. of light petroleum (b.p. 40–60°) by vigorous shaking in 250 ml. conical separating funnels, the aqueous layers being discarded.

At 20–5° the distribution coefficient of indole between light petroleum and water is 5.01 (Scott, 1960), and at equilibrium 91% of the indole is extracted into the organic solvent layer by a single extraction. Careful standardization of the volumes of aqueous and organic phases during extraction is essential; a 25% deviation from the volume of aqueous phases gives rise to a 2.2% deviation in the indole content of the organic phase. During extraction from aqueous solutions containing denatured protein, slow phase separation can be avoided by filtration through a plug of glass wool, which is then washed (Scott, 1960).

Production and measurement of colours. To indole contained in 40 ml. of light petroleum is added 10 ml. of Ehrlich reagent or *p*-dimethylaminocinnamaldehyde reagent. After vigorous shaking, the coloured aqueous layer is drained into tubes and centrifuged. The coloured solutions are transferred to spectrophotometer tubes by Pasteur pipette and the colour densities read in a Unicam SP. 1400 diffraction-grating spectrophotometer, usually with pure reagent as blank. Colours are read 15–20 min. after addition of Ehrlich reagent at 560 m μ , and after exactly 30 min. at 640 m μ with *p*-dimethylaminocinnamaldehyde reagent. Readings are referred to a calibration curve constructed by

extracting known amounts of indole in aqueous solution by the above-described procedure.

Ehrlich reagent is prepared by dissolving 16 g. of *p*-dimethylaminobenzaldehyde in 1250 ml. of ethanol and then adding 320 ml. of concentrated hydrochloric acid. The reagent is 67 mM with respect to *p*-dimethylaminobenzaldehyde and approx. 2N with respect to hydrochloric acid.

p-Dimethylaminocinnamaldehyde reagent was finally prepared by dissolving 2.34 g. in 780 ml. of ethanol, adding 100 ml. of concentrated hydrochloric acid and making up to 1000 ml. with water. This reagent is 13.4 mM with respect to *p*-dimethylaminocinnamaldehyde, and approximately N with respect to acid.

Standard solutions of indole were prepared by dissolving freshly sublimed samples in ethanol to give a 1% (w/v) solution. The ethanolic solution was then diluted, usually to 10 μ g. of indole/ml., with water.

RESULTS AND DISCUSSION

Substitution of an equimolar amount of *p*-dimethylaminocinnamaldehyde in the preparation of Ehrlich reagent yielded a highly coloured solution which absorbed strongly between 550 and 650 m μ . To facilitate zero adjustment for the reagent blank, the *p*-dimethylaminocinnamaldehyde concentration was decreased to one-tenth of this concentration. With 10 ml. of this diluted reagent, approx. 10 μ g. of indole was extracted from light petroleum by the usual procedure. The absorption spectrum of the dark-green colour thus produced showed a strong absorption peak at 646 m μ [absorption maximum was observed to be 625 m μ with a Unicam SP. 500 spectrophotometer (Scott, 1960)], although the absorption of this peak rapidly decreased with time. Colour fading was not dependent on light.

In an attempt to measure the rates of colour formation and decay 9 ml. of *p*-dimethylaminocinnamaldehyde reagent was added to 1 ml. of aqueous indole solution (10 μ g. of indole) contained in a spectrophotometer tube. Absorption at 640 m μ reached a maximum after 7 min., and the colour was stable over the next 15 min. The improved colour stability was concluded to be a result of decreased concentration of acid during colour development. The absorption spectrum of the colour, plotted during the period of colour stability, is shown in Fig. 1.

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Effect of concentration of hydrochloric acid on colour development and stability. A series of reagent mixtures were prepared, with a standard solution of *p*-dimethylaminocinnamaldehyde in ethanol, such that, after dilution with various proportions of concentrated hydrochloric acid and water, the overall concentration of reagent was 6.7 mM. The development and decay of colours obtained by extracting approx. 20 μ g. of indole from light petroleum with the series of reagents, by the usual procedure, are shown in Fig. 2. The conclusions drawn from these and other results were that as the concentration of hydrochloric acid in the reagent increases, the rate of development of maximum colour intensity increases but the final colour intensity and the time period over which the colour is stable decrease. In the present work, it was decided to compromise between a rapid development of colour, high colour intensity and stability. The reagent used in all further work was approx. N with respect to hydrochloric acid.

Effect of concentration of p-dimethylaminocinnamaldehyde on colour density. This factor was investigated with 5 μ g. of indole, as it was amounts of

indole of this order that were of interest. The maximum colour density was measured at 640 $m\mu$ with various concentrations of *p*-dimethylaminocinnamaldehyde after periods of exactly 30 min. colour development (Table 1). From these results it appeared that, under the conditions used, a suitable reagent concentration was 13.4 mM with respect to *p*-dimethylaminocinnamaldehyde.

Sensitivity of p-dimethylaminocinnamaldehyde and Ehrlich reagents. A calibration curve was constructed by light-petroleum extraction of between

Fig. 1. Absorption spectrum of *p*-dimethylaminocinnamaldehyde colour with indole. To 9 ml. of *p*-dimethylaminocinnamaldehyde reagent, 6.7 mM, and 2N with respect to acid, was added 10 μ g. of indole in 1 ml. of aqueous solution. The spectrum was plotted between 7 and 15 min. after mixing, with water replacing indole solution in the blank. The absorption maximum was observed at 646 $m\mu$.

Fig. 2. Effect of acid normality on colour development and stability. Aqueous indole solutions, 20 μ g. in 20 ml., were extracted with 20 ml. of light petroleum. Colours were developed with 10 ml. of *p*-dimethylaminocinnamaldehyde reagent, 6.7 mM, at various acid normalities: 0.3N, Δ ; 0.5N, $\circ$; 1.0N, $\square$; 1.7N, $\bullet$; 2.3N, $\times$. Colour development was followed at 640 $m\mu$.

Table 1. *Effect of p-dimethylaminocinnamaldehyde on colour density*

Aqueous solutions of indole, 5 μ g. in 20 ml., were extracted with 40 ml. of light petroleum, and colours were developed with *p*-dimethylaminocinnamaldehyde reagent, N with respect to acid, as described in the text.

Final concn. of reagent (mM)	<i>E</i> at 640 $m\mu$ after 30 min.
6.7	0.193
13.4	0.264
20.1	0.266

1 and 10 $\mu\text{g.}$ of indole from 20 ml. of aqueous solution by the standard procedure. Colour development and measurement, with *p*-dimethylaminocinnamaldehyde reagent, were carried out as described above. The results are shown in Fig. 3. Included in Fig. 3 is part of a linear calibration curve obtained with Ehrlich reagent. For amounts of indole below 10 $\mu\text{g.}$ of the latter reagent gives

Fig. 3. Indole calibration curves with *p*-dimethylaminocinnamaldehyde reagent and Ehrlich reagent. The indole-extraction procedure, also the production and measurement of colours, were carried out as described in the Experimental section. Extinction values: with *p*-dimethylaminobenzaldehyde (Ehrlich) reagent, ●; with *p*-dimethylaminocinnamaldehyde reagent, ○.

poorly reproducible results, whereas *p*-dimethylaminocinnamaldehyde reagent has been found to give approx. $\pm 5\%$ accuracy for amounts of indole above 1 $\mu\text{g.}$ If *p*-dimethylaminocinnamaldehyde reagent containing hydrochloric acid at an overall normality of less than 1 is used, and with longer standard development times, the method is more than 2.2 times as sensitive as the method with Ehrlich reagent.

p-Dimethylaminocinnamaldehyde reagent has been used in these laboratories for the assay of tryptophanase activity during purification studies. The increased sensitivity of the reagent was found to be valuable when limited amounts of fractions from *Escherichia coli* extracts were available. The reagent has also been used (T. A. Scott, unpublished work) for the indirect assay of small amounts of tryptophan by initial enzymic breakdown to indole.

SUMMARY

1. Details are presented of a modified procedure for the extraction of indole into light petroleum from aqueous tryptophanase reaction systems.
2. The use of *p*-dimethylaminocinnamaldehyde as a reagent for indole is described, and factors affecting the rate of colour formation, colour density and stability are described.
3. The quantitative use of this reagent is compared with that of Ehrlich reagent. The sensitivity increase under the conditions selected is greater than 2.2, and the reagent is capable of assaying amounts of indole greater than 1 $\mu\text{g.}$ contained in 20 ml. of aqueous solution.

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