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THE USE OF FLUORIMETRY IN THE ESTIMATION OF NATURALLY-OCCURRING INDOLES IN PLANTS

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Abstract—Methods have been developed for the use of spectrophotofluorimetry in the identification and estimation of indole compounds in plant extracts. The sensitivity of the method is of the same order as that of the biological assays coupled with chromatography currently in use but it has a much greater accuracy (1–2%). Provided fairly pure samples of individual compounds can be obtained, they can be identified with reasonable confidence when present in fractions of a μg by using a combination of properties such as activation and fluorescent spectral characteristics, pH–fluorescence relationships, solvent partition and R_f values on paper and on columns of cellulose or cellulose derivatives. The application of these methods to the analysis of extracts of white cabbage is described.

INTRODUCTION

FOR OVER thirty years biological assays have been the mainstay of all work on the naturally-occurring auxins. Over the whole of this period they have remained the most sensitive of tests and for the first twenty years the solution of the enigma of the respective roles of auxin A, auxin B and 'heteroauxin' (i.e. indole-3-acetic acid) was sought in various empirical tests involving the use of such assays. With the advent of paper chromatography the biological assay of auxins was adapted to this new analytical technique, but only when large quantities of extracted plant material were used could colorimetric tests be applied for the more precise characterization of the active substances thereby disclosed.

The pea root segment test[1] is about the most sensitive of the biological assays and can detect as little as 10^{-5} μg of indole-3-acetic acid (IAA) but cannot distinguish between an auxin *sensu stricto* and a growth inhibitor. One of the most sensitive of the true auxin assays for use with chromatography involves segments of *Avena* internodes[2] and can detect IAA in concentrations of approximately 0·01 μg/ml. Compared with these the most sensitive colorimetric tests for indoles are very poor. Thus tests involving ferric chloride (e.g. Salkowski) cannot detect concentrations much less than 2 μg/ml.[3] In the Ehrlich reaction the sensitivity is a little greater (colours can be obtained with as little as 1·0 μg/cm^2 of chromatographic paper). But biological assays are completely non-specific and, using them alone, the only indication of the nature of an active substance on a chromatogram is its R_f value. Furthermore, biological assay, unless many times replicated, is very imprecise and, with the usual variation of response of biological material, cannot measure auxin concentrations with an error much less than $\pm$ 30–40%. The unsatisfactory nature of the present situation has been succinctly stated by Steward and Shantz[4] who say: "It is no longer particularly instructive to

[1] L. J. AUDUS and R. THRESH, *Physiol. Plant.* **6**, 451 (1953).
[2] J. P. NITSCH and C. NITSCH, *Plant Physiol.* **31**, 44 (1956).
[3] J. W. MITCHELL and R. C. BRUNSTETTER, *Botan. Gaz.* **100**, 802 (1939).
[4] F. C. STEWARD and E. M. SHANTZ, *Ann. Rev. Plant Physiol.* **10**, 381 (1959).

multiply examples of these alleged growth-promoting 'substances' which are designated solely by areas on the chromatogram... unless the responsible compounds can be isolated and identified."

In work on the extremely small quantities of auxins present in plant roots, we have tried to develop methods combining the sensitivity of bioassay with the specificity of spectrometry. The intense fluorescence of indole compounds in u.v. light seemed to be most promising. Such methods have already been used with paper chromatography, both before and after chemical treatments, for the qualitative demonstration of these compounds.[5–8] Quantitative estimations on paper chromatograms have also been attempted. By determining the fluorescent emission intensity, quantities of IAA as small as 0·5 μg per spot have been measured but the relationship between concentration and emission is non-linear and accuracy is not very great below 10 μg per spot.[9] However, this method is of very little value as it stands with plant extracts which are usually loaded with fluorescent compounds of all kinds, very few of which may be indole auxins. Unless we can control the wavelength of the irradiating u.v. light, and unless the wavelength of the emitted fluorescent light is precisely determined, there is no hope of identifying, let alone measuring, indole compounds on such irradiated chromatograms. Promise of increased sensitivity and specificity came from a report of work by Ebert[10] who formed a fluorescent derivative of IAA by heating it in a solution of $CuSO_4$ in concentrated H_2SO_4. Such promise has not been realized, as will be seen later from the results of a critical investigation of Ebert's technique.

Progress in the application of fluorimetry to the identification and estimation of plant indoles has been hindered by the lack of suitable instruments for the easy determination of both the excitation and the fluorescent spectra over the whole u.v. and visible range. In recent years this has been made possible by the development of the high pressure Xenon arc-lamp which emits a continuous spectrum from 200–500 mμ. In 1955 two instruments for recording these spectra were developed around such light sources. They were the Aminco–Bowman and the Farrand spectrophotofluorimeters, both based on a prototype described by Bowman *et al.*[11] The Aminco–Bowman instrument, which has been used in the work to be described, is capable of recording within the course of a few minutes both the excitation and the emission spectra of 2 ml of a given solution. Under given conditions the wavelengths of the maxima of these two spectra are characteristic of a compound and provide two very important parameters for its identification. The method is very sensitive for indole compounds and it is possible to make quantitative estimations of IAA with a practical lower limit of sensitivity of about 0·0175 μg/ml at pH 5·0. One very great advantage that fluorimetry has to offer over other chemical or physico-chemical techniques at present available is that estimates can be made on the solution in which biological assay is subsequently carried out. This eliminates errors which are unavoidably associated with the replications of chromatograms needed for other types of parallel estimations and introduces a very satisfying precision in the comparison of responses of any particular component of a chromatogram.

Some attention has already been given to the fluorescent behaviour of indole compounds. Thus aromatic amino-acids including tryptophan have been studied by Duggan and

[5] J. B. JEPSON and B. J. STEVENS, *Nature*, **172**, 772 (1953).
[6] S. P. SEN and A. C. LEOPOLD, *Physiol. Plant.* **7**, 98 (1954).
[7] H. LINSER and O. KIERMAYER, *Biochem. Biophys. Acta* **21**, 382 (1956).
[8] J. A. BENTLEY, K. R. FARRAR, S. HOUSLEY, G. P. SMITH and W. C. TAYLOR, *Biochem. J.* **64**, 44 (1956).
[9] R. MAVRODINEAU, W. W. SANFORD and A. E. HITCHCOCK, *Contrib. Boyce Thompson Inst.* **18**, 167 (1955).
[10] A. V. EBERT, *Phytopathol.* **24**, 216 (1955).
[11] R. L. BOWMAN, P. A. CAULFIELD and S. UDENFRIEND, *Science* **122**, 32 (1955).

Udenfriend.[12] Data for the excitation and emission maxima of certain indole compounds of biological interest are given by Duggan *et al.*[13] The effects of pH are described by Udenfriend, Weissbach and Clark,[14] and White,[15] and the effects of various organic solvents by van Duuren.[16] But indole compounds occurring in plants have been little studied and therefore the first step in these investigations has been to characterize the fluorescent behaviour of as many such compounds as could be obtained in pure form in order to evaluate the relevant parameters for their identification and estimation in plant extracts. This paper will also describe the use of fluorimetry in the analysis of extracts of white cabbage, a material known to contain relatively large quantities of indole auxins.

METHODS OF SPECTROPHOTOFLUORIMETRY

The construction and operation of the Aminco–Bowman spectrophotofluorimeter have been fully described by Udenfriend[17] and will not be repeated here.

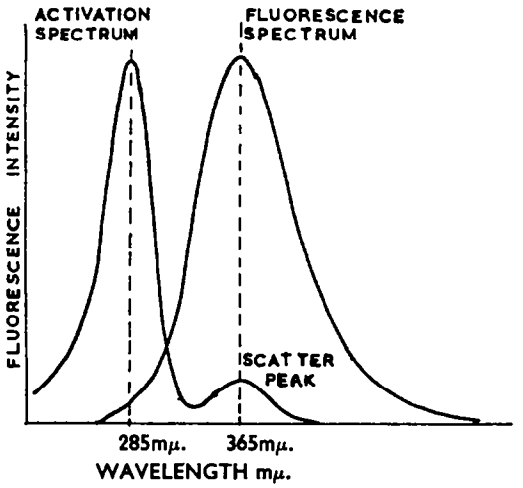

FIG. 1. FLUORESCENCE SPECTRA OF INDOLE-3-ACETIC ACID.
(1 μg/ml in phosphate–citrate buffer at pH 5·0.)

Excitation and emission spectra for indoleacetic acid (IAA) are shown in Fig. 1. It will be seen that the excitation spectrum shows a second small peak at the wavelength of the fluorescent maximum. This is a spurious effect due to the scattering of the activating light by the test solution, and it determines the lower limit of sensitivity of the method (see Fig. 2).

The instrument has wavelength scales for both monochromators but these were found not to be correctly aligned with the grating and needed careful calibration. This was done by using a solution of quinine sulphate in decinormal sulphuric acid at a concentration of

[12] D. E. DUGGAN and S. UDENFRIEND, *J. Biol. Chem.* **223**, 313 (1956).
[13] D. E. DUGGAN, R. L. BOWMAN, B. B. BRODIE and S. UDENFRIEND, *Arch. Biochem. Biophys.* **68**, 1 (1957).
[14] S. UDENFRIEND, H. WEISSBACH and C. T. CLARK, *J. Biol. Chem.* **215**, 337 (1955).
[15] A. WHITE, *Biochem. J.* **71**, 217 (1959).
[16] B. L. VAN DUUREN, *J. Org. Chem.* **26**, 2954 (1961).
[17] S. UDENFRIEND, *Fluorescence Assay in Biology and Medicine*, Academic Press, New York and London (1963).

1 μg/ml. By using scattered light reflected from a piece of ground-glass in the cell, it was found that the misalignment errors were uniform over both the wavelength scales and so correction could be made by the addition of a constant factor to any wavelength reading.

For quantitative estimates of known compounds calibration curves are needed. Figure 2 shows such a curve for indole-3-acetic acid from measurements done on two separate occasions. The curve is virtually linear over the whole range used. The lower limit of sensitivity set by solution scatter is about 2×10^{-8} M and concentrations up to approximately 3×10^{-5} M can be measured before the curve starts to flatten as a result of self-quenching. This has the virtue of the biological assay in that it can be used to measure concentrations over at least three

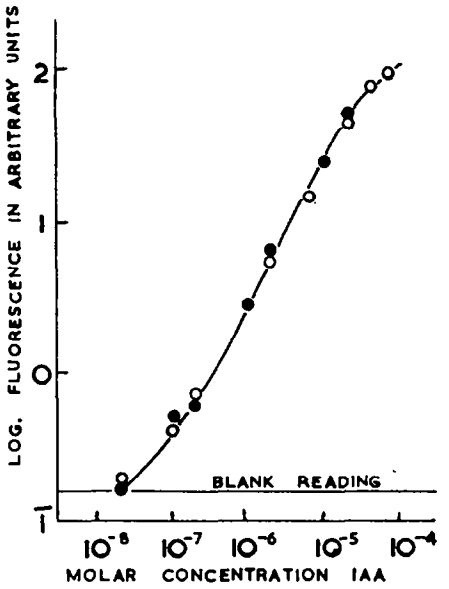

FIG. 2. THE RELATIONSHIP BETWEEN CONCENTRATION OF INDOLE-3-ACETIC ACID (IN PHOSPHATE–CITRATE BUFFER AT pH 5) AND FLUORESCENCE INTENSITY IN ARBITRARY GALVANOMETER READINGS.

The open and closed circles correspond to two separate sets of measurements made on two different occasions.

orders of magnitude. It also has a sensitivity equal to most biological assays but surpasses all of them in its very much greater accuracy.

The instrument possesses certain basic limitations. In theory, the precision with which the spectra can be traced and the accuracy with which the peak wavelength can be measured are determined by the width of the slits in the optical system; the narrower the slits the more precise the wavelength determination but the lower the sensitivity. In practice, however, the lower limit of accuracy depends on the mechanical scanning device and the accuracy with which the wavelength scale can be read. For all practical purposes this is 5 mμ. In this work therefore relatively wide $\frac{3}{16}$ in. slits have been used throughout for both incident and emergent beams to provide maximum sensitivity with very low concentrations of fluorescent compounds met in this work.

A further limitation to accurate quantitative analysis lies in the inherent instability of the Xenon arc-lamp. The arc has a tendency to wander, which means that the total activating flux

incident on the test solution will vary. This is minimized by using the relatively large slits. Even over short time intervals this variation can amount to as much as 5%. However, the error can be minimized by making rapid alternate comparisons of test solution and standard. The order of accuracy of replicated readings is shown by the calibration curve of IAA which was constructed from two sets of observations made on different occasions. New lamps with much more stable performance have recently been developed.

A large number of factors may affect the intensity of the fluorescent light emitted from an irradiated solution. The most obvious one is the fluorescence of other compounds present as impurities; even though their excitation and fluorescent spectra may be widely spaced from those of the substance under consideration, yet if their fluorescence is high the 'tails' of their spectra may so interfere with measurement that considerable inaccuracies will result. Even if the contaminating substance is not itself fluorescent its *absorption* spectrum may overlap that of the emission spectrum of the test substance to such an extent that the intensity of the emitted light is much reduced. This means that the solution being analysed should contain as little as possible of contaminating material; the method is virtually useless with crude extracts of plant material or even with eluates from one-way chromatograms. Such extracts must first be subjected to very extensive purification by solvent partition and several stages of chromatography.

A further source of difficulty arises in the use of organic solvents to extract plant material. In the reduction of such extracts to minimum volume, fluorescent solvent impurities often increase in concentration with the extracted compounds. Even ethanol specially purified for absorption spectroscopic measurements contained such impurities and was therefore rejected for our work. Methanol and ether redistilled over $FeSO_4$ are the only two solvents we found to be satisfactory in this respect.

Working with very dilute solutions of the order of 10^{-7} to 10^{-6} M also involves inaccuracies which we feel may not have been appreciated by the users of the relatively inaccurate techniques of bioassay. For example, substances may be strongly adsorbed to the surfaces of the containing vessel and in spectrometric measurements a considerable fraction may be oxidized or photo-decomposed. The results of such changes in plant extracts will be described in a subsequent paper. Udenfriend[17] in his recent manual has fully discussed these problems.

FLUORESCENT CHARACTERISTICS OF SYNTHETIC INDOLE COMPOUNDS

1. *Fluorescence Characteristics and Classification*

The excitation and fluorescence spectra for all the indole compounds so far studied are single peaked as shown in Fig. 1 for IAA. Twenty-five compounds of possible significance as plant hormones or hormone precursors have been investigated; twenty-two have been classified in a two-way table based on the wavelengths of their excitation and fluorescence optima (Table 1). Three (viz. indole-3-acrylic acid, indole-3-aldehyde and indole-3-acetaldehyde) which showed no fluorescence whatever under these conditions, could not be so classified. Although the range of wavelengths covering these maxima are not very wide, it is possible to distinguish twelve separate groups. Eight contain only one compound and each of these can therefore be distinguished from all the others on fluorescent parameters alone (e.g. IAN). Other groups contain more than one compound; for example the three homologous compounds, indole-3-acetic, indole-3-propionic, and indole-3-butyric acids, are indistinguishable. But even very dissimilar compounds may have the same spectral maxima. Thus there are seven compounds out of those examined which have an excitation maximum

TABLE 1. CLASSIFICATION OF INDOLE COMPOUNDS ACCORDING TO FLUORESCENCE CHARACTERISTICS*

Fluorescence maxima	Excitation maxima				
	275 mμ	280 mμ	285 mμ	290 mμ	295 mμ
340 mμ	Gramine				5-OH Tryptophan 5-OH Tryptamine
345 mμ		Indole-3-carboxylic acid (B)			
350 mμ	Indole (Aa)	Indole-3-acetonitrile (Aa)			5-OH Indole-3-acetic acid
355 mμ		Indole-3-acetamide, Tryptamine (Aa) Indole-3-acetylglycine, Indole-3-acetylasparagine, Indole-3-acetylglutamine (Ab) Tryptophan, N-methyl-tryptophan (C)			
360 mμ		N-acetyl-tryptophan (Ab) Indole-3-lactic acid (Ad) Tryptophan (above pH 4) (C)			
365 mμ		Tryptophol (Ac)	Indole-3-propionic acid (Ae) Indole-3-butyric acid (Af) Indole-3-acetic acid (Ag)	Indole-2-carboxylic acid	
370 mμ		Skatole (Ac)			

* The letters in parenthesis show the pH–fluorescence behaviour as grouped in Table 2.

at 280 mμ and a fluorescent maximum at 355 mμ, including such dissimilar substances as indole-3-acetylasparagine and N-methyl-troptophan. It is clear then that other distinguishing properties are needed to sub-divide these groups further. Differences in the pH–fluorescence relationships offer such a possibility.

2. *pH Relationships*

A change in the pH of the medium can affect the fluorescence of a compound in three ways. First, it can cause a shift in the wavelength of the fluorescence maximum; secondly, it can change the intensity of the fluorescence without affecting its wavelength; thirdly, it can cause a combination of these two effects.

Taking first the wavelength shift, the effect of high acidity on the fluorescent maxima of the 5-hydroxy indoles is well known. Udenfriend, Weissbach and Clark[14] showed that in neutral

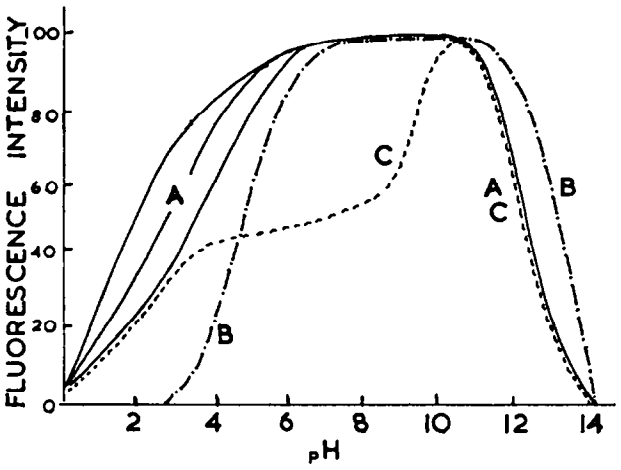

FIG. 3. TYPES OF pH–FLUORESCENCE CURVES SHOWN BY THE EIGHTEEN INDOLE COMPOUNDS OF TABLE 2. The curves have all been scaled to the same maximum so that their shapes can be accurately compared. All measurements were made at wavelengths corresponding to maximum excitation and fluorescence.

or slightly acid solutions such compounds fluoresce with a maximum at 330 mμ, while in strong hydrochloric acid a new fluorescent peak appears at 550 mμ. With the exception of tryptophan, this wavelength shift of the fluorescence maximum does not occur with any other of the compounds investigated here. With tryptophan the fluorescence maximum in neutral solutions is 355 mμ, and this shifts to 360 mμ in alkaline solutions above pH 10. This effect has also been noted by Gally and Edelman.[18]

The only remaining effect of pH which might be of use in further discriminating these indole compounds is that on fluorescence intensity. Here the differences in behaviour between compounds is so marked that it can be used for discrimination.

It has not been possible to cover the whole of the wide pH range required by the use of one buffer mixture. This unavoidable use of completely different buffers disclosed the fact that the fluorescent behaviour of certain compounds may be considerably affected by one or other component of the buffer system. For example, the quenching effects of electron-donating or electron-accepting ions on the fluorescence of organic molecules is well known and is

[18] J. A. GALLY and G. M. EDELMAN, *Biochim. Biophys. Acta* **60**, 499 (1962).

discussed by Bowen and Wokes.[19] Such an effect showed up in four of the compounds we studied, namely the three 5-hydroxy indole compounds and indole-2-carboxylic acid. No significant effects due to buffer could be detected in any of the remaining eighteen compounds which gave smooth curves with no discontinuities over the whole pH range.

The curves shown by these eighteen compounds are of three major types which are illustrated in Fig. 3 where fluorescence is expressed as a percentage of that at the maximum point of the curve. In this way direct comparisons of the pH effects can be made. The majority of the compounds exhibit type A curve, which is flat-topped and shows little change in the maximum fluorescence over the pH range 6 to 11. In more alkaline solutions the fluorescence falls away steeply along a curve which is identical for all the compounds in this group. Fluorescence disappears completely at pH 14. In more acid solutions below pH 6 there is a

TABLE 2. CLASSIFICATION OF COMPOUNDS ON pH/FLUORESCENCE CHARACTERISTICS

The numbers against the compounds of curve type A* represent the values of pH at which the declining curve of fluorescence in acid solutions reaches a value half that of the maximum. The compounds are arranged in ascending order of this half maximum pH value.

Curve type A	Curve type B	Curve type C
a. Indole, Indole-3-acetonitrile, Indole-3-acetamide, Tryptamine } 2·0	Indole-3-carboxylic acid	Tryptophan, N-methyl-tryptophan
b. N-acetyl-tryptophan, Indole-3-acetyl glycine, Indole-3-acetyl glutamine, Indole-3-acetyl asparagine } 2·7		
c. Tryptophol, Skatole } 3·0		
d. Indole-3-lactic acid, 3·4		
e. Indole-3-Propionic acid, 3·7		
f. Indole-3-butyric acid, 3·8		
g. Indole-3-acetic acid, 3·9		

* See Fig. 3.

similar fall away and fluorescence again virtually disappears at pH 0·1. Compounds in this group, however, do differ in the positioning of this decline in acid solutions, the pH at which half maximum fluorescence is attained varying between 2 and 3·9. These values are recorded against the respective compounds in Table 2. It will be seen that on this basis the fourteen compounds fall into seven groups. Although in some cases the differences are small, for example between the propionic, butyric and acetic acids, and could not therefore be relied upon to discriminate between these compounds especially in partially purified extracts, yet the differences in others are so large as to allow easy discrimination, for example between indole-3-acetonitrile and the three acids mentioned above.

A curve of type B is exhibited by only one compound studied, indole-3-carboxylic acid. It is similar to the A-type curve but differs in that the declines in fluorescence occur at higher pH values.

[19] E. J. BOWEN and F. WOKES, *Fluorescence of Solutions*, Longmans Green, London (1953).

The third type, C, is shown by two compounds, tryptophan and N-methyltryptophan, which possess identical and characteristic curves. There is a sharp peak of fluorescence at pH 11 from which the curve declines on the alkaline side like the type A curve. On the acid side of the peak it declines in two steps, reaching a plateau of about 45% maximum fluorescence between pH 4 and 7 and then declining to zero at pH 0·1 similar to the type A curves. The curve is so characteristic that it has been of considerable value in identifying tryptophan in plant extracts. This work will be described in a subsequent paper.

This classification based on pH fluorescence relationships has been included in Table 1, where the curve type is marked in brackets after the compounds. It will be seen that the two

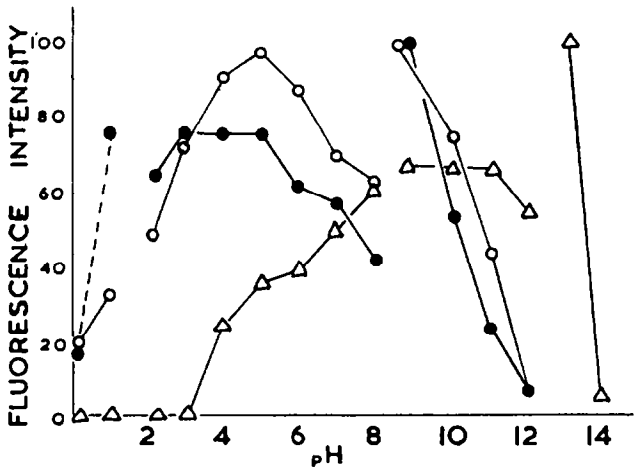

FIG. 4. pH–FLUORESCENCE CURVES OF 5-HYDROXY-TRYPTAMINE (●), 5-HYDROXYTRYPTOPHAN (○) AND INDOLE-2-CARBOXYLIC ACID (△).

The curves have all been scaled to the same maximum so that their shapes can be accurately compared. The pH range was achieved by the use of acids and buffers as follows:

pH 0·1 and 1·0	N and N–10H_2SO_4.
pH 2·2–8·0	Phosphate–citrate buffer (mixtures of 0·2 M Na_2HPO_4 and 0·1 M citric acid).
pH 8·9–12·1	Sorenson's (Walbum) NaCl glycine–NaOH buffer.
pH 13·2 and 14	N–10 and N NaOH.

major groups of compound, i.e. those activated at 280 mμ and fluorescing at 355 and 360 mμ respectively, are thus subdivided into a total of six groups. Working with pure compounds it should be possible to separate these six groups from each other on fluorescent behaviour alone, but in practice, with somewhat contaminated solutions and extracts, it might not be possible to distinguish between Aa and Ab or between Ab and Ad although the A- and C-type curves are completely distinguishable.

As we have mentioned previously, there are four compounds among those studied which show anomalous behaviour in that the fluorescence seems to change with the buffer used. This shows up as a major discontinuity in the pH–fluorescence curves at points where a change is made from one buffer to another. In the case of the three 5-hydroxy-indole compounds this takes place between pH 8 and pH 8·9 where the buffer changes from phosphate–citrate to NaCl–glycine–NaOH, (see Figs. 4 and 5). The evidence suggests that the effect might be due

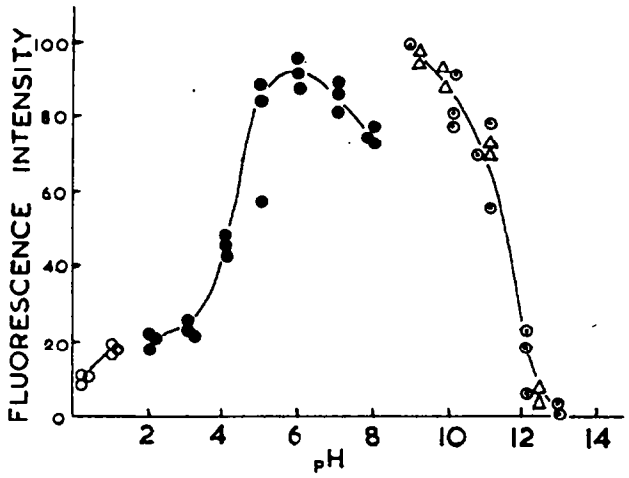

FIG. 5. pH–FLUORESCENCE CURVES FOR 5-HYDROXY-INDOLE-3-ACETIC ACID.

○ In N and N–10 H_2SO_4.
● In phosphate–citrate buffer (mixtures of 0·2 M Na_2HPO_4 and 0·1 M citric acid).
⊙ In Sorensen's (Walbum) NaCl–glycine–NaOH buffer.
△ In Borate–NaOH buffer

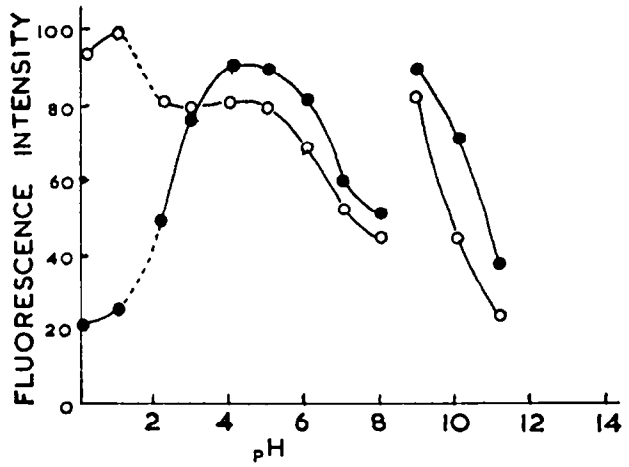

FIG. 6. pH–FLUORESCENCE CURVES FOR TYROSINE (●) AND PHENOL (○).

The pH range was achieved by the use of acids and buffers as follows:

pH 0·1 and 1·0	N and N–10H_2SO_4.
pH 2·2–8·0	Phosphate–citrate buffer (mixtures of 0·2 M Na_2HPO_4 and 0·1 M citric acid.
pH 8·9–11.1	Sorensen's (Walbum) NaCl–glycine–NaOH buffer.

to a quenching of the fluorescence in the phosphate–citrate buffer between pH 6 and 8, since two separate buffers, viz. borate–NAOH buffer and NaCl–glycine–NaOH buffer, which were used for 5-OH-indole-3-acetic acid over pH range 9 to 13, both gave virtually the same curve (Fig. 5). If this is true it would seem to be correlated with increased phosphate concentrations and thus probably due to the phosphate ion. The effect seems to be associated particularly with hydroxylation in the benzene ring, since it is also shown by the amino acid tyrosine and even by phenol itself (see Fig. 6).

The fourth compound showing anomalous behaviour is indole-2-carboxylic acid with a smooth curve over the whole range from pH 0·1 to pH 12 using three different buffers but with a marked discontinuity at higher pH's where NaOH alone is used (Fig. 4).

These phenomena need further investigation and may eventually be of help in the identification of these four and other similar compounds.

3. *Ebert's Technique Re-investigated*

In 1955 Ebert[10] claimed that by heating IAA with a solution of copper sulphate in concentrated sulphuric acid a very highly fluorescent derivative was formed which provided a

TABLE 3. COMPARISON OF THE PROPERTIES OF INDOLE-3-ACETIC ACID AND ITS REACTION PRODUCT IN ACID $CuSO_4$

Solution components			Colour of solution		Fluorescent intensity after boiling	
IAA	$CuSO_4$	H_2SO_4	Before boiling	After boiling	Indole-3-acetic acid*	Reaction product†
+	−	−	Clear	Clear	30	0
+	+	+	Pink	Pink	0	5·4
+	+	−	Pale blue	Pale blue	12·6	0
+	−	+	Clear	Pink	0	1·6

* Measured at activation max. 285 mμ and fluorescent max. 365 mμ.
† Measured at activation max. 395 mμ and fluorescent max. 485 mμ. (Secondary activation max. at 340 mμ not used.)

relatively specific and highly sensitive technique for the estimation of indole-3-acetic acid. It was decided to re-investigate this promising technique to see whether it could be improved upon by the use of our more flexible instrument. Ebert's instructions were followed carefully but in addition IAA was heated alone with copper sulphate and with sulphuric acid separately. The reaction mixture consisted of 10 ml of IAA solution (5·5 μg/ml) with 2 ml of 0·05 M $CuSO_4$ and 5 ml conc. H_2SO_4. The mixture was heated for 5 min in boiling water, cooled for 3 min under running water and then allowed to stand in the dark until room temperature was reached.

Table 3 shows that this treatment yields a pink IAA reaction product with fluorescent characteristics completely different from those of IAA. However, it is also produced by heating with H_2SO_4 alone, although the presence of Cu greatly increases its production. Judging by the disappearance of its characteristic fluorescence, IAA is completely destroyed in the reaction. However, contrary to Ebert's claims, the fluorescence of the reaction product, even at its maximum concentration, is only about one-sixth that of IAA.

This discrepancy no doubt arises from the fact that Ebert used a fluorimeter with completely different spectral responses. His instrument, a Lumetron colorimeter, Model E, of the Photovolt Corporation, New York, embodies a mercury vapour lamp which emits little light below 300 mμ, the emission spectrum having peaks at 313 mμ (minor) and 366, 405 and 436 mμ. These peaks are nearer the excitation maxima of the reaction product (340 and 395 mμ) than that of IAA at 285 mμ. Similarly the relative spectral sensitivity of the photocell (barrier layer type) in the Lumetron colorimeter at the fluorescent maxima of IAA (365 mμ) is 32% whereas at the fluorescent maxima of the reaction product (485 mμ) it is 95%. His instrument was therefore relatively well suited for detecting and measuring the fluorescence of the reaction product but very poorly suited for that of IAA.

However, reaction products can sometimes be much more fluorescent than a parent compound and can therefore greatly enhance sensitivity. This approach has been used in the detection and estimation of dihydroxyphenylalanine in bean roots, to be described in a subsequent paper.

THE CORRELATION OF FLUORIMETRIC ESTIMATION WITH BIOASSAY

The applicability of these fluorimetric methods to auxin assay has been tested by making careful comparisons of fluorimetric measurements and bioassays on one and the same solution. This has been done by analysing firstly mixtures of known indole compounds, and secondly extracts of white cabbage whose indole auxins have been much studied.[20, 21]

1. *Synthetic Compounds*

In Fig. 7 are plotted the results of the bioassay and fluorimetric estimation of five dilutions of an IAA solution ranging from 10^{-8} to 10^{-4} M. It will be seen that the limits of sensitivity of the *Avena* first internode bioassay (ca. 10^{-8} M) is only slightly lower than the limit in the fluorimetric estimation (2×10^{-8} M). In the lower range of IAA concentration the two estimations are linearly related one to the other but in higher concentrations, which are optimal and supra-optimal for growth, the fluorescent estimation can still be used with precision and accuracy whereas the bioassay cannot.

The same kind of curve has been constructed for the two isomers of tryptophan and their racemic mixture. The results of the two assays are plotted in Fig. 8 for three concentrations of tryptophan, viz. 10^{-4}, 10^{-5}, and 10^{-6} M. Again we see that there is a close linear relationship between the two types of measurement but the curves do not fall one on the other as might have been expected. Whereas both isomers and their racemic mixture in the same total concentration show identical fluorescence behaviour, the growth responses to D-tryptophan are slightly less than half the responses to L-tryptophan in all concentrations. If tryptophan activity in these tests is due only to its conversion in the tissue to indole-3-acetic acid, as is usually supposed, these results imply that this conversion takes place with different efficiencies with the two isomers. However, judging from the growth activities of the isomers relative to that of IAA it is clear that tryptophan conversion can only be partial. One very interesting feature of the curves is the fact that the racemic mixture produces a consistently greater growth response than the L-isomer. This implies that conversion of one or other of the isomers proceeds much more rapidly in the presence of the other than in its absence. In this way the total amount of IAA produced by the racemic mixture would be very much greater than that

[20] E. R. H. JONES, H. B. HENBEST, G. E. SMITH and J. A. BENTLEY, *Nature*, **169**, 485 (1952).
[21] H. LINSER, O. KIERMAYER and E. YOUSSEF, *Planta* **52**, 173 (1958).

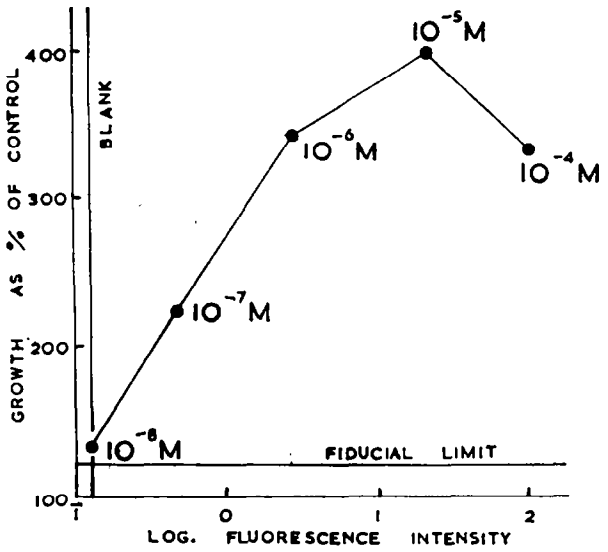

FIG. 7. THE RELATIONSHIP BETWEEN BIOLOGICAL (*Avena* FIRST INTERNODE SEGMENT) AND FLUORESCENCE ASSAY FOR FIVE CONCENTRATIONS OF INDOLE-3-ACETIC ACID IN PHOSPHATE–CITRATE BUFFER AT pH 5.

The concentrations of the auxin are marked against the corresponding points of the curve.

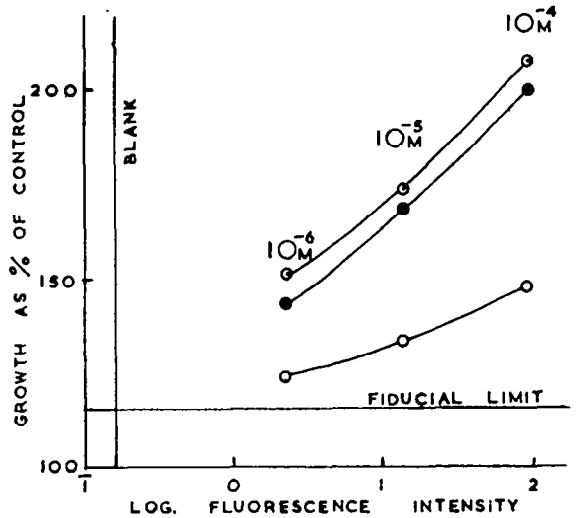

FIG. 8. THE RELATIONSHIP BETWEEN BIOLOGICAL (*Avena* FIRST INTERNODE SEGMENT) AND FLUORESCENT ASSAY FOR THREE CONCENTRATIONS OF D- (○), L- (●) AND DL-TRYPTOPHAN (⊙) IN PHOSPHATE–CITRATE BUFFER AT pH 5.

The concentrations of tryptophan are marked against the corresponding points of the curve.

produced by twice the amount of the more active L-isomer. These speculations need checking by metabolic studies in which the spectrophotofluorimeter could be very useful.

It should also be possible to use this comparison of biological and fluorimetric analyses to

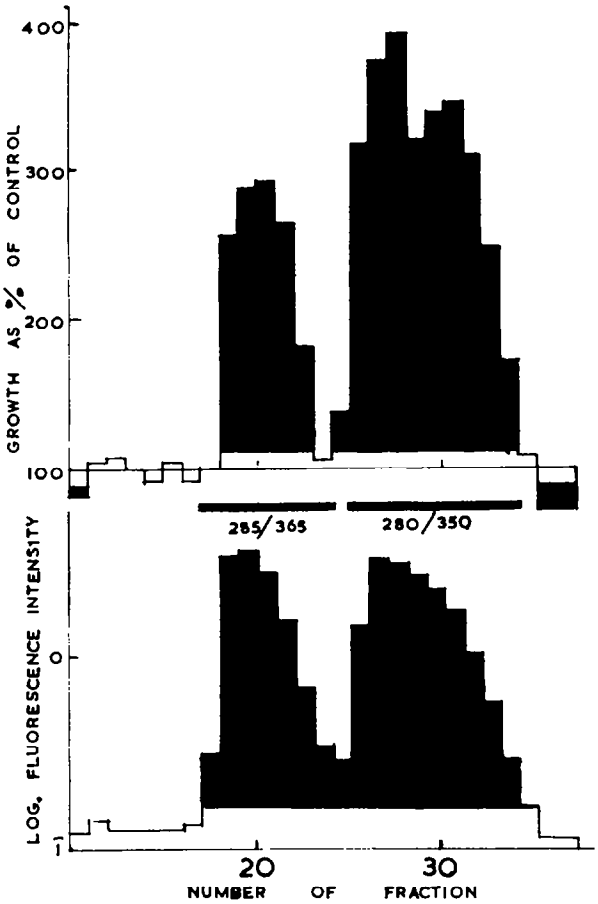

FIG. 9. CHROMATOGRAMS OF A MIXTURE OF INDOLE-3-ACETIC ACID AND INDOLE-3-ACETONITRILE SEPARATED ON A CELLULOSE POWDER COLUMN RUN WITH DISTILLED WATER AS SOLVENT.

Avena first internode segment assays (top chromatogram) and fluorescent estimations (bottom chromatogram) were performed on the same 2·5-ml fractions from the column. The fluorescence was determined directly on the fraction while the bioassay was carried out after buffering to pH 5 with phosphate–citrate buffer and adding sucrose to a concentration of 2%. The activation and fluorescent peak wavelengths are marked over the corresponding fractions of the chromatogram. The white areas along the base lines of these chromatograms represent ± the fiducial limits of the first internode assay and the level of the black reading of the fluorescence determination respectively.

determine which isomeric form of tryptophan is present in plant tissues. Microbiological determination has already been employed to this end in tomato root extracts by Thurman and Street[22] who claim that tryptophan in that tissue is in the L-form.

The two assays have further been used in conjunction to test the efficiency of a cellulose

[22] D. A. THURMAN and H. E. STREET, *J. Exp. Bot.* **11**, 118 (1960).

powder column to separate an artificial mixture of IAA and IAN. A solution containing equal amounts of IAA and IAN in water was put on a cellulose column 56 cm by 1 cm, which had previously been thoroughly washed with distilled water to remove all traces of fluorescent materials. The column was eluted with water and successive 2·5 ml fractions collected. Each fraction was analysed in the fluorimeter and assayed by the *Avena* first internode test after the addition of the appropriate buffer. The results shown in Fig. 9 indicate the precision with which bioassay and fluorimetric results can be compared for one and the same fraction. The curves show that the two results are very closely comparable and demonstrate the slightly greater sensitivity of the bioassay but the much greater precision of the fluorimetric analysis.

2. *Application to the Analysis of Cabbage Extracts*

Freshly harvested leaves from the colourless inside of white cabbage were macerated in a blendor in 80% methanol at room temperature. From this filtered and centrifuged extract

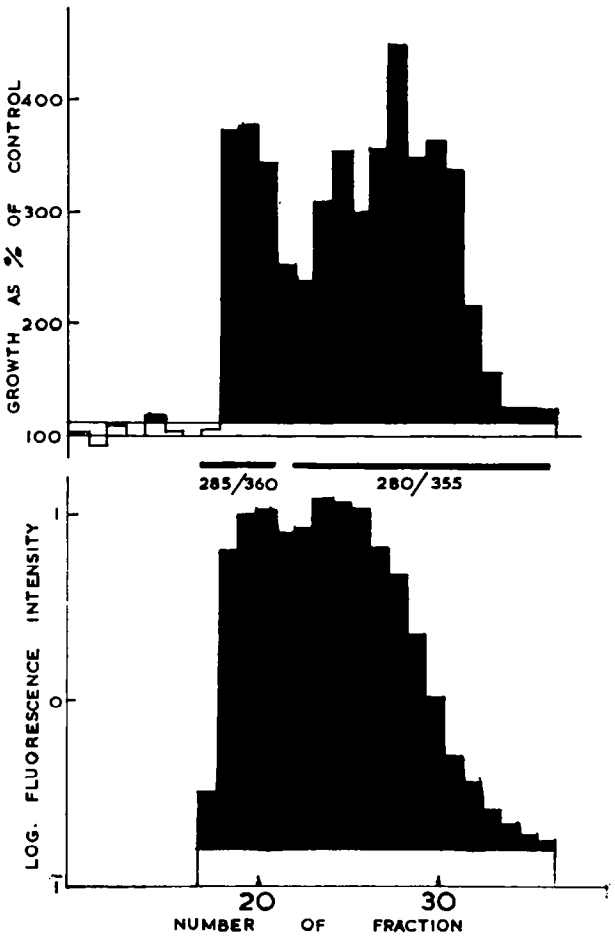

FIG. 10. CHROMATOGRAM OF THE WATER-SOLUBLE COMPONENT OF A METHANOL EXTRACT OF 2 g OF WHITE CABBAGE LEAF TISSUE ON A CELLULOSE POWDER COLUMN WITH DISTILLED WATER AS SOLVENT. *Avena* first internode assay (top chromatogram) and fluorescent estimation (bottom chromatogram). Other details as for Fig. 9.

the water-soluble, ether-soluble acid and ether-soluble neutral components were separated by the traditional solvent partition techniques (see Methods). After concentration under vacuum at 25° aliquots of each component equivalent to 2 g fresh weight of the original cabbage leaf were applied to the tops of washed cellulose powder columns. These columns were then run with distilled water; the first ten 2·5-ml fractions of each run were inactive but were collected and used as controls for the bioassay and blanks for the fluorimetric analysis. A total of 38 fractions were collected, this removing most of fluorescent materials from the

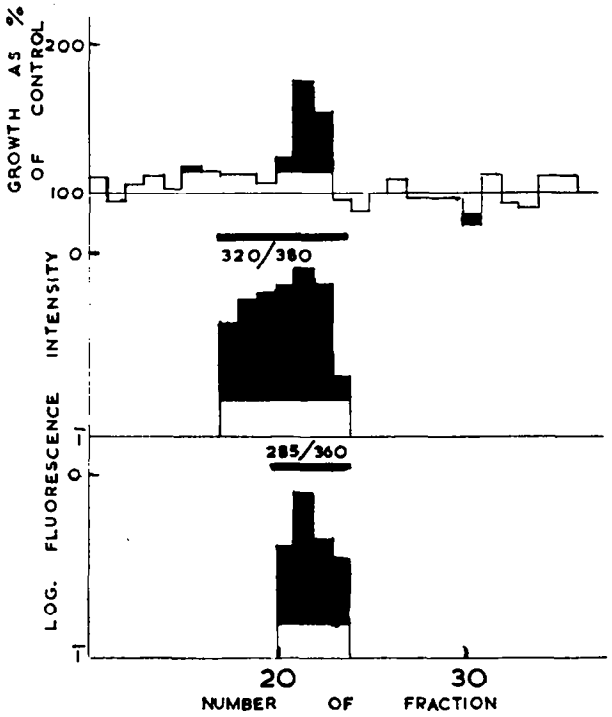

FIG. 11. CHROMATOGRAM OF THE ACID ETHER SOLUBLE COMPONENT OF A METHANOL EXTRACT OF 2 g OF WHITE CABBAGE LEAF TISSUE ON A CELLULOSE POWDER COLUMN WITH DISTILLED WATER AS SOLVENT.

Top—*Avena* first internode assay; middle—estimation of substance with excitation max. 320 mμ and fluorescent max. 380 mμ; bottom—estimation of substance with excitation max. 285 mμ and fluorescent max. 360 mμ. Other details as for Fig. 9.

column. The *Avena* first internode assay and the fluorimetric analysis of these chromatograms for the three components are shown in Figs. 10, 11 and 12.

Taking first the water-soluble material, it will be seen that two clearly defined bands separate out on the column. Both are highly growth active. The first one appearing in fractions 18 to 24 show an excitation maximum of 285 mμ and a fluorescence maximum of 360 mμ which do not correspond precisely with any of the pure compounds (See Table 1). The other component appearing in fractions 22 to 37 shows an excitation maximum at 280 mμ and a fluorescent maximum at 355 mμ corresponding to the groups in Table 1 into which tryptophan, indole-3-acetylasparagine, etc. fall. Although the fluorescence measurements show but one peak in these fractions (fractions 23 and 24) which correspond to a similar auxin

activity peak, the biological assay exhibits a second much higher peak with a maximum in fraction 28, where the fluorescence intensity is falling rapidly away. It seems quite clear that in these fractions (27–31) there is an additional highly active auxin which is non-fluorescent.

One interesting fact should be mentioned at this point and this is that the first component in fractions 18 to 24 exhibits continuously increasing fluorescence during the course of the

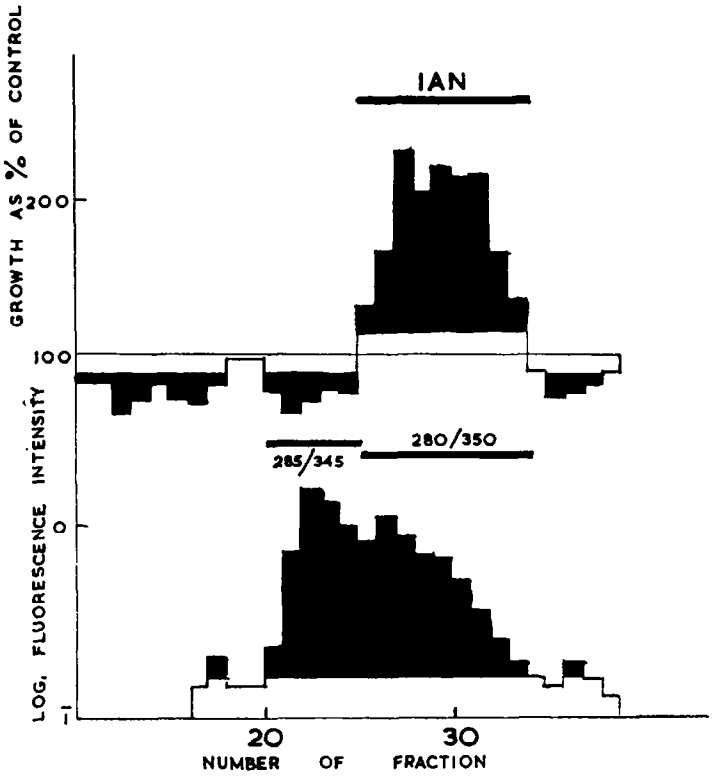

FIG. 12. CHROMATOGRAM OF THE NEUTRAL ETHER-SOLUBLE COMPONENT OF A METHANOL EXTRACT OF 2 g OF WHITE CABBAGE LEAF TISSUE ON A CELLULOSE POWDER COLUMN WITH DISTILLED WATER AS SOLVENT.

Avena first internode assay (top chromatogram), fluorescent estimations (bottom chromatogram). Other details as for Fig. 9.

readings. This must be due to some photochemical production of a highly fluorescent compound. Careful study of the spectral characteristics of the solution did not reveal the appearance of any other excitation or fluorescent maxima and this suggests one of two possibilities. Either the highly fluorescent product possesses identical spectral characteristics with the component from which it is formed, or the component is formed from a non-fluorescent precursor which has the same partition coefficient on the cellulose column. The first of these possibilities seems the most likely. Photochemical decomposition of this kind has been observed with β-(3,4-dihydroxyphenyl)alanine (DOPA) isolated from the roots of *Vicia faba* but the product here was easily distinguished from DOPA by having quite different spectral characteristics.

The analysis of the acid ether-soluble material (Fig. 11) shows up the presence of one auxin in fractions 20 to 23 and two fluorescent components which overlap it. The first having an excitation maximum of 320 mμ and a fluorescence maximum of 380 mμ occurs between fractions 18 and 24. This corresponds to no known indole compound and is apparently not an auxin. The other component which occurs in fractions 20 to 23 and which coincides very closely with the growth activity has an excitation maximum at 285 mμ and a fluorescent maximum at 360 mμ which coincides very nearly, although not precisely, with the indole-3-acetic acid group of Table 1. Although this fraction has not been further investigated, it seems probable that this latter component is indeed indole-3-acetic acid, since it runs at almost the same position on the column as synthetic IAA (see Fig. 9). It should be noted that not so much reliance can be placed on the use of the wavelength of the fluorescent maximum for identification in these solutions since the presence of another fluorescent component excited at (and

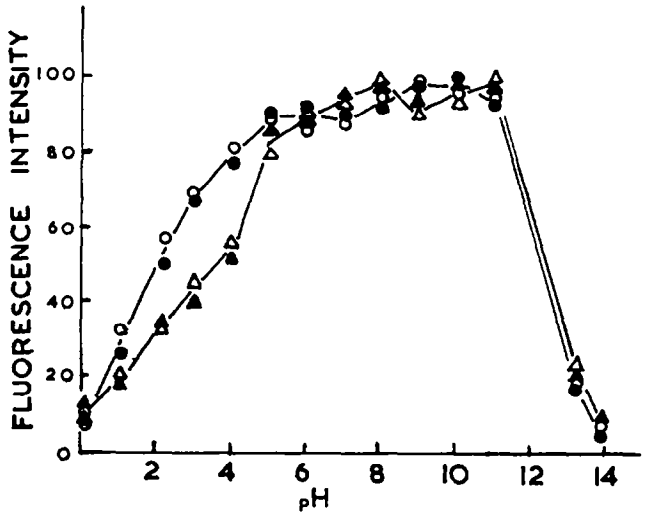

FIG. 13. pH–FLUORESCENCE CURVES FOR THE UNKNOWN NEUTRAL ETHER-SOLUBLE AUXIN FROM WHITE CABBAGE LEAF TISSUE (●) AND ITS HYDROLYSIS PRODUCT (▲) COMPARED WITH THOSE OF SYNTHETIC INDOLE-3-ACETONITRILE (○) AND INDOLE-3-ACETIC ACID (△).

All curves are scaled to the same maximum to allow a precise comparison of their shapes.

therefore absorbing at) 320 mμ and fluorescing at 380 mμ could interfere with the exact determination of the wavelength of the maximum fluorescence of the IAA component. Further chromatographic separation would be needed to use this parameter with confidence.

The analysis of the neutral ether-soluble fraction (Fig. 12) reveals two major components. The first running between fractions 20 and 24 has no growth activity and has an excitation maximum at 285 mμ and a fluorescent maximum at 345 mμ. The second component which is highly growth active and which runs at exactly the same position on the chromatogram as synthetic IAN shows fluorescent properties which are characteristic of that compound. This substance has been further investigated by collecting together all relevent fractions and carrying out two further tests on them. In one, aliquots have been taken and the pH–fluorescence characteristics determined; in the other a portion of the material has been heated at 100° for 30 min in 7 N NAOH. By this latter treatment IAN would be completely converted into IAA. The material from this treatment was extracted with ether at pH 3·0 and its pH–

fluorescence characteristics determined. Other properties of this component and its hydrolysis product were also determined. These included R_f values on paper chromatograms run in isopropanol–water (4:1), Ehrlich colour reactions and behaviour on cellulose derivative ion exchange columns (see Fig. 13 and Table 4). It will be seen that in all these tests the properties of the unknown component corresponds precisely with those of IAN, and the properties of its hydrolysis product corresponds precisely with IAA. There seems little doubt therefore that the growth-active component of the neutral ether fraction is indole-3-acetonitrile. At this point

TABLE 4. PROPERTIES OF THE NEUTRAL ETHER-SOLUBLE AUXIN OF WHITE CABBAGE LEAVES AND ITS HYDROLYSIS PRODUCT

Substance	Fluorescence		R_f*	Ehrlich colour reaction	Anion column (DEAE cellulose)	Cation column (cellulose phosphate)
	λ-Activation maximum mμ	λ-Fluorescence maximum mμ				
Unknown auxin	280	350	0·76	Pink	Not retained	Not retained
IAN	280	350	0·76	Pink	Not retained	Not retained
Hydrolysis product	285	365	0·88	Purple	Retained	Not retained
IAA	285	365	0·90	Purple	Retained	Not retained

* Paper, Whatman's No. 2: Solvent, isopropanol–water, 4:1.

however a warning must be sounded that the IAN is not necessarily a constituent of undamaged cabbage tissue. As Virtanen[23] and his colleagues have recently shown, IAN can be one of a number of indole compounds produced by the enzymatic breakdown in crushed cabbage tissue of glucobrassicin, an indole glycoside. Since no precautions were taken in our extraction to inactivate enzymes in advance, IAN may easily have arisen in this way.

GENERAL DISCUSSION

The investigations which have been presented in this paper show that without doubt fluorescence analysis is potentially a very powerful tool in the identification and estimation of plant indoles and one which possesses a sensitivity only fractionally less than that of the bioassay most commonly used in current researches. Coupled with refined methods of chromatographic separation, whereby fractions containing only one fluorescent compound can be prepared, this method far surpasses in accuracy any other method at present in use. It has however serious limitations which are linked to its very great sensitivity. They arise from the fact that in work with plant extracts which have to be subjected to considerable concentration and analysed by complicated solvent partition and chromatographic techniques the range of suitable solvents is very considerably restricted. So far, only three, namely distilled water, methanol, and ether, have been obtained sufficiently free from fluorescent impurities that they can be used for extraction. Similar, although not so serious, limitations are imposed on the choice of solvents for chromatography. Here however concentration of impurities does not take place during the procedure and therefore one can tolerate a rather higher level of fluorescent contaminants.

[23] I. A. VIRTANEN, *Arch. Biochem. Biophys.* Supp. I, 200 (1962).

MATERIALS AND METHODS

The Preparation of Solutions and Buffers

The stock solutions of all synthetic compounds were 2×10^{-4} M. Relatively insoluble compounds were first dissolved in 1 ml of methanol and then diluted with distilled water. Solutions for analysis were prepared by diluting stock solutions with the appropriate buffer in two stages to a final concentration of 2×10^{-6} M. 2 ml of this final solution were used for fluorimetric examination. The buffers used were McIlvaine's citrate–phosphate buffer for the pH range 2·2 to 8·0 and Sorenson's (Walbum) NaCl–glycine–NaOH buffer for the pH range 8·9 to 12·1. For some observations a borate–NaOH buffer was occasionally used for additional observations over the pH range 9·2 to 12·4. For very low pH of 0·1 and 1·0, solutions of normal and decinormal sulphuric acid were used while pH 13·2 and 14 were obtained with decinormal and normal solutions of NaOH.

When investigating plant extracts, 0·25- and 0·025-ml aliquots of extract were diluted directly with 2·25 or 2·5 ml of buffer, thus allowing a study of the pH/fluorescent intensity relationships of the extract even with the small quantities of the original solutions available, for example, 5 ml fraction from a column chromatogram.

The Bioassay Technique

Avena satina var. Victory I (Svalof) grain were soaked in tap water for 2 hr in darkness. They were sown in sterilized sand in glass half-bricks, covered with an inverted half-brick and germinated in complete darkness at 25° for 72 hr. 3-mm segments of the first internode were cut for assay with a double-bladed cutter at a distance of 2 mm below the coleoptilar node. They were soaked for 1 hr on butter muslin, stretched on the surface of distilled water in order to equilibrate them before the test.

All bioassay solutions were made up in 0·01 M phosphate–citrate buffer at pH 5·0 and contained 2% sucrose. Each assay sample was of ten segments grown in 1 ml of the bioassay solution for 24 hr in complete darkness at 25° under conditions of gentle agitation.

Final segment lengths were recorded by mounting segments on a slide in batches and directly printing on bromide paper in a photographic enlarger at a magnification of ×4.

Solvent Partition Techniques

Three ml of the solution of the concentrated extract were acidified to pH 3·0 with 0·1 N phosphoric acid and then shaken with 15 ml of re-distilled ether in a stoppered bottle. The ether was then decanted and the extraction repeated four more times, each with 15 ml of fresh ether. On each occasion mechanical shaking continued for 15 min. The water solution remaining contains the water-soluble fraction. The pooled ether extracts were then shaken for 15 min with 15 ml of 5% sodium bicarbonate solution and this was repeated four more times, and the bicarbonate fractions pooled. This procedure removes the ether-soluble acid substances leaving behind in the ether the neutral ether fraction. The sodium bicarbonate solution was then acidified to pH 3 with 75% syrupy phosphoric acid and this solution shaken four times with 15 ml of freshly distilled ether each time for 15 min. The pooled ether solutions constitute the acid ether fraction. The two ether solutions were both dried over sodium sulphate overnight and then evaporated to dryness under vacuum at 25°. The water-soluble fraction was taken to pH 7 with saturated barium hydroxide and the resultant precipitate spun down at 4000 rev/min. The supernatant was then taken down to dryness under vacuum at 26°.

Acknowledgements—We wish to acknowledge with grateful thanks the generosity of Dr. J. B. Jepson of the Courthauld Institute of Biochemistry, Middlesex Hospital, London, for gifts of the following indole compounds used in this work: indole-3-acetylglycine, indole-3-acetylasparagine, indole-3-acetylglutamine, indole-3-lactic acid, indole-3-carboxylic acid, N-methyl-tryptophan, N-acetyl-tryptophan, 5-OH-tryptophan, 5-OH-tryptamine and 5-OH-indole-3-acetic acid. We would also like to express our appreciation of considerable help in the bioassay of white cabbage leaf extracts by Mrs. Mira Nešković when in the department as a guest from the University of Belgrade, Yugoslavia.