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FLUORIMETRIC ESTIMATION OF THE INDOLE GLUCOSINOLATE GLUCOBRASSICIN

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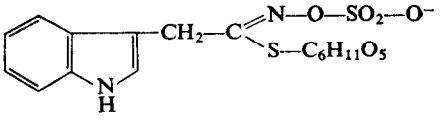
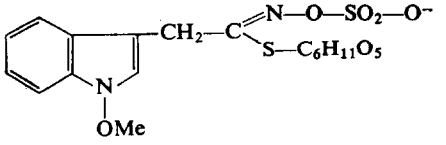
Abstract—A method of fluorimetric estimation of the indolic glucosinolate glucobrassicin is described. Glucobrassicin reacts with *p*-dimethylaminobenzaldehyde in HCl and gives a green–blue fluorescence excited by u.v.-radiation. The optimum conditions of the reaction and of measuring the intensity of fluorescence by means of a spectrophotometer with a fluorimetric adapter, a mercury vapour lamp and a hydrogen discharge lamp have been investigated. The products of the reaction and conditions of measurement have been controlled by spectrofluorimetry. The resulting complex of glucobrassicin with *p*-dimethylaminobenzaldehyde is activated by u.v. radiation with peaks at 320 and 396 nm and the fluorescence spectrum has a peak at 490 nm.

INTRODUCTION

THE INDOLE thioglucosinolate, glucobrassicin (I) was isolated by Gmelin and Virtanen[1] when examining *Brassica* species for the precursor of the thiocyanate ion which exerts a goitrogenic effect. Studies of the occurrence and biosynthesis of I in the vegetable kingdom showed that it is present in plants belonging to many other genera of the Brassicaceae (or Cruciferae)[2] as well as in the Resedaceae, Capparidaceae and Tovariaceae.[3] Besides I, its *N*-methoxyderivative neoglucobrassicin (II),[4] has also been found to be widespread, and the two compounds appear to be the most frequent glucosinolates encountered in plants.



Glucobrassicin (I)



Neoglucobrassicin (II)

The physiological function of I in plants is related to its participation in indole[5–7] and sulphur[7,8] metabolism. I is formed from tryptophan in brassica;[7,9] its decomposition by the myrosinase enzyme complex results in the formation of ascorbigen, the auxin indolylacetonitrile and thiocyanate.[1,7,9]

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[1] R. GMELIN and A. I. VIRTANEN, *Ann. Acad. Sci. Fenn.*, Ser. A, II, *Chemica* 107 (1961).
[2] M. KUTÁČEK and K. OPLIŠTILOVÁ, *Fiziol. Rastěnij* **11**, 867 (1964).
[3] H. SCHRAUDOLF, *Experientia* **21**, 520 (1965).
[4] R. GMELIN and A. I. VIRTANEN, *Acta Chem. Scand.* **16**, 1378 (1962).
[5] M. KUTÁČEK, *Abstracts, VIth Int. Congr. Biochem.* **1**, 10 (1964).
[6] M. KUTÁČEK and Ž. PROCHÁZKA, Régulateurs de la croissance végétale, Centre Nat. des Recherches Scientifiques, Paris (1964).
[7] H. SCHRAUDOLF and F. BERGMANN, *Planta* **67**, 75 (1965).
[8] M. KUTÁČEK, J. SPÁLENÝ and K. OPLIŠTILOVÁ, *Experientia* **22**, 24 (1966).
[9] M. KUTÁČEK, Ž. PROCHÁZKA and K. VEREŠ, *Nature* **194**, 393 (1962).

I can be estimated either by determining the quantity of thiocyanate ions which are split off by myrosinase[10] or by estimating its indole content. Earlier,[6] we suggested the polarographic estimation of I on the basis of its indolic character and in our present work we are describing its fluorimetric estimation.

The fluorimetry of indoles can be carried out by using a sufficiently intensive source of short-wave u.v. radiation (*ca.* 285 nm) when it is possible to excite fluorescence and to estimate the indoles directly without a chemical transformation.[11,12] The direct measurement of fluorescence requires a spectrofluorimeter with a xenon arc-lamp as a source of radiation. However, the assay of certain fluorescent derivatives does not require such apparatus, since the activation of fluorescence occurs at higher wave-lengths where radiation even from a mercury vapour lamp is sufficient. Earlier, we developed fluorimetric methods for the estimation of indoles after reaction with the Procházka formaldehyde agent.[6,13–16] The Procházka reagent unfortunately proved to be unsuitable for the fluorimetric estimation of I. However, the reaction of I with *p*-dimethylaminobenzaldehyde (PDAB) does give a suitable fluorescent derivative.[6] We have adapted this method of estimation for an apparatus with a mercury vapour lamp as a source of radiation and also for a spectrophotometer with an adapter for fluorimetry with a hydrogen lamp as a more suitable source of u.v. radiation.

RESULTS

Conditions for the Fluorimetric Determination of Glucobrassicin

(*a*) *Effect of* p-*dimethylaminobenzaldehyde and of* HCl *concentration.* The optimum concentration of PDAB has been determined separately for the various apparatus. For the work with Spekol (measured at 435 nm wavelength) PDAB concentration of 0·02% (Fig. 2) gave best results in combination with the optimum HCl concentration of 12% in the final sample (Fig. 1). For the work with the spectrophotometer CF 4 and the spectrofluorimeter, the optimum PDAB concentration was 0·1% with the HCl concentration of 13% in the final sample.

(*b*) *Effect of boiling time on the reaction intensity and the stability of fluorescence.* The heating of the sample in a boiling water bath increased the fluorescence intensity. The optimum heating time was in the range of 10–12 min. During the first hour after the end of the reaction, the fluorescence intensity declined only moderately, during the next 2 hr a stronger drop has been observed, whereafter the fluorescence intensity nearly stabilized itself. The course of this reaction in the light is faster than that of samples kept in the dark.

(*c*) *Activating energy and fluorescence spectra of glucobrassicin.* The construction of Spekol allows the activation by discontinuous radiation, as the apparatus is provided with a mercury vapour lamp HQE 40 emitting a relatively long-wave u.v. line spectrum with maxima at 365, 404·7, 407·8, 435·8, 577 and 579 nm. Only the fluorescence spectrum has been determined; the peaks correspond to the spectral lines of the mercury vapour lamp. The maximum fluorescence intensity has been measured (Fig. 3) at 435 nm. The spectrophotometer CF 4

[10] N. MICHAJLOVSKIJ, *Chemické zvesti*, in print, Symposium on Isothiocyanates, Smolenice, October 15–17 (1964).
[11] D. BURNETT and L. J. AUDUS, *Phytochem.* **3**, 395 (1964).
[12] B. B. STOWE and J. F. SCHILKE, Régulateurs de la croissance végétale, Centre Nat. des Recherches Scientifiques, Paris (1964).
[13] M. VALENTA, M. KUTÁČEK and V. ŠANDA, *Rostlinná výroba, Praha* **35**, 1487 (1962).
[14] J. KOŠTÍŘ and M. VALENTA, *Rostlinná výroba, Praha* **36**, 981 (1963).
[15] M. VALENTA, Ž. PROCHÁZKA and M. KUTÁČEK, *Rostlinná výroba, Praha* **37**, 1207 (1964).
[16] M. KUTÁČEK, J. NOVÁKOVÁ and M. VALENTA, *Flora* **153**, 54 (1963).

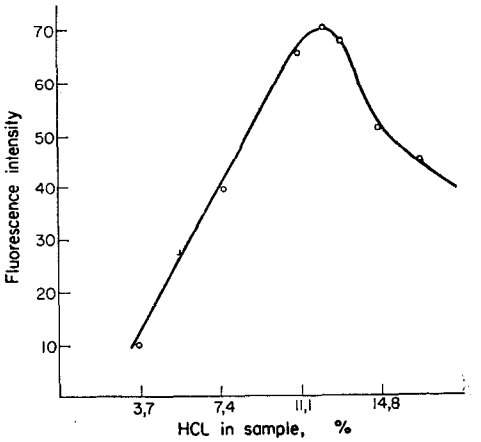

FIG. 1. THE RELATIONSHIP BETWEEN THE CONCENTRATION OF HCl AND THE FLUORESCENCE INTENSITY AFTER REACTION OF I WITH PDAB MEASURED ON SPEKOL (ZEISS JENA).

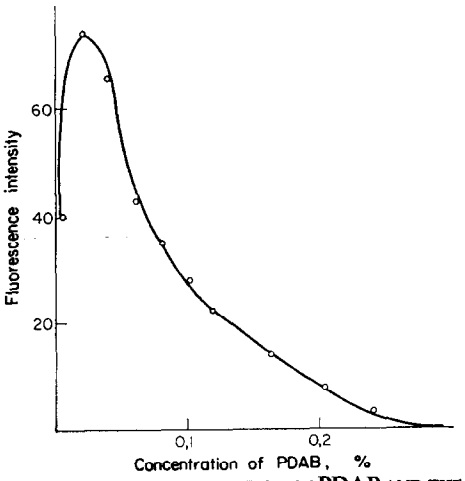

FIG. 2. THE RELATIONSHIP BETWEEN THE CONCENTRATION OF PDAB AND THE FLUORESCENCE INTENSITY OF I MEASURED ON SPEKOL (ZEISS JENA).

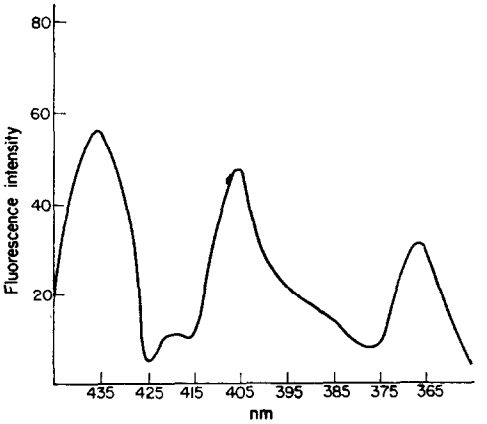

FIG. 3. FLUORESCENCE SPECTRUM OF I WITH PDAB MEASURED WITH SPEKOL, (ZEISS JENA).

with a more convenient hydrogen discharge tube allows, by exchanging the corresponding filters, to alter the range of activating radiation; its intensity was sufficient even in the short-wave region. The optimum filter had a transmission peak at 330 nm. The peak of the fluorescence spectrum was at 490 nm (Fig. 4). The optimum conditions for using both apparatus have been controlled by a spectrofluorimeter (Figs. 5 and 6).

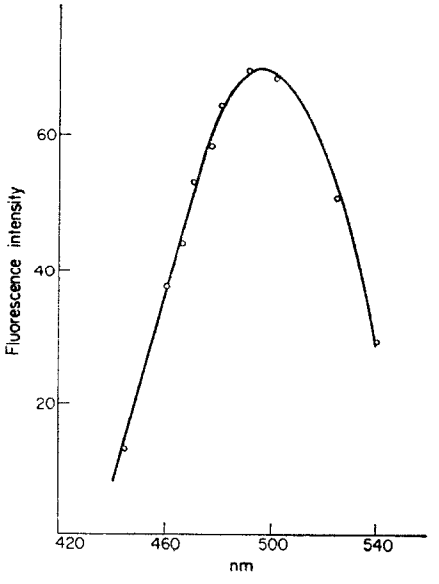

FIG. 4. FLUORESCENCE SPECTRUM OF I WITH PDAB ACTIVATED BY RADIATION AT 330 nm WAVELENGTH, MEASURED ON THE SPECTROPHOTOMETER CF 4 (OPTICA PILANO).

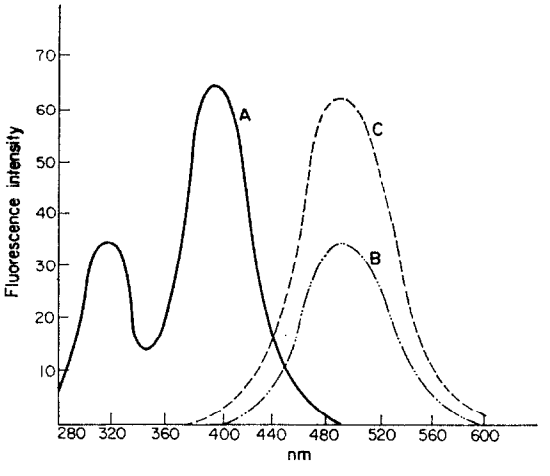

FIG. 5. ACTIVATION AND FLUORESCENCE SPECTRA OF I MEASURED ON THE SPECTROFLUORIMETER (FARRAND)

PDAB II Reagent: curve A: activation spectrum when measuring fluorescence intensity at 490 nm; curve B: fluorescence spectrum-activation at 320 nm; curve C: fluorescence spectrum-activation at 400 nm.

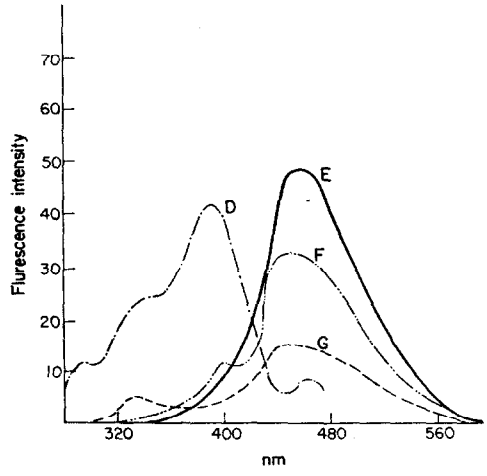

FIG. 6. ACTIVATION AND FLUORESCENCE SPECTRA OF I.

PDAB I reagent: curve D: activation spectrum when measuring fluorescence intensity at 490 nm; curve E: fluorescence spectrum-activation at 400 nm; curve F: fluorescence spectrum at activation 355 nm; curve G: fluorescence spectrum-activation at 300 nm.

In the modification suited for the measurement on Spekol (PDAB reagent I) we found a distinct peak of the activating spectrum at 390 nm and two lower peaks at 347 and 302 nm. The fluorescence spectra corresponding to the activation peaks had a maximum fluorescence intensity in the range of 440 and 460 nm. In the modification suited for the spectrophotometer CF 4 (PDAB reagent II), the activating spectrum was characterized by two peaks at 320 and 396 nm, the second one being more conspicuous. The fluorescence spectra corresponding to both activation peaks had the optimum value at 490 nm, which corresponds to the results obtained with the spectrophotometer CF 4.

(*d*) *Effect of the glucobrassicin concentration of fluorescence intensity.* The calibration curve using the method for Spekol approximates to a straight line and can be used for direct measuring of I from 2 μg/ml upwards; even at 30 μg/ml the dependance has a linear character. The calibration curve obtained when using the spectrophotometer is also linear and I can be determined from 0·4 μg/ml upwards.

Fluorescence of Various Indole Derivatives and Effects of Various Additives

The fluorescent reaction with PDAB has been tested with a number of indoles (Table 1). PDAB yielded a stronger fluorescence with indolylacetic acid, with indolylpyruvic acid, indolylacetonitrile, indolylacetamide; fluorescence with a maximum intensity was yielded by I. II also gives a fluorescent reaction with PDAB; it can therefore be determined by this fluorimetric method. *p*-Dimethylaminocinnamaldehyde is not a suitable reagent for the fluorescent reaction. Addition of alcohol to an aqueous sample containing PDAB diminishes the fluorescence intensity. The effect of the addition of methanol, ethanol, propanol and propyleneglycol was tested. The replacement of HCl by 70% $HClO_4$ has little effect on the fluorescence intensity.

TABLE 1. FLUORESCENCE OF INDOLE DERIVATIVES AFTER REACTION WITH *p*-DIMETHYLAMINOBENZALDEHYDE

Indole derivative	Fluorescence intensity
Indole	3
Indolyl-3-aldehyde	6
Indolyl-3-carboxylic acid	6
Indolyl-3-acetic acid	12
Indolyl-3-butyric acid	9
Indolyl-3-pyruvic acid	21
Indolyl-3-glyoxalic acid	3
Tryptophol	6
Indolyl-3-acetonitrile	12
Indolyl-3-acetamide	18
Tryptamine	3
Tryptophan	nil
Indolylacetylaspartic acid	3
5-Hydroxytryptophan	6
5-Hydroxyindolyl-3-acetic acid	9
Ascorbigen	11
Glucobrassicin	51
Glucoiberin	nil

The Resulting Method of Fluorimetric Estimation of Glucobrassicin with p-*Dimethylaminobenzaldehyde*

(*a*) *Treatment of plant material and chromatography.* Whole plant material is put into three times its wt. of boiling methanol. After boiling for 2–3 min, it is cooled and left for 2 hr at 5°. The plant material is then homogenized and the volume of the resulting brei is measured, being made up to a definite volume with methanol, if necessary. After centrifuging, approx. 250 μl of the methanolic extract is applied to a chromatographic paper. After developing with *n*-butanol–HOAc–H_2O (4:1:2), the position of I is determined by treatment of a control standard with the Procházka or *p*-dimethylaminocinnamaldehyde reagent and the sample material is cut out from the chromatogram. The paper, cut into small pieces, is extracted with 3 ml H_2O for 3 min in a boiling water bath. After cooling and filtering, 2 ml of the extract were used for the determination. Standard curves for I were prepared in a similar way.

(*b*) *Method of fluorimetric estimation of glucobrassicin using the Spekol apparatus.* The 2 ml extract and 1 ml of reagent I (0·06% PDAB) are heated together for 10 min at 100° and the fluorescence intensity is measured (at 435 nm) on the cooled solution within 1 hr.

(*c*) *Method of fluorimetric estimation of glucobrassicin using the spectrophotometer CF* 4. The 2 ml extract, 0·5 ml reagent II (1% PDAB) and 2·5 ml 6 N HCl are heated for 10 min at 100°, cooled to 20° and the fluorescence intensity is measured within 1 hr. The activation wavelength, 330 nm, is applied and the fluorescence intensity is measured at 490 nm.

Dispersion of the Results and Estimation of Glucobrassicin Recovery in Plant Material

The error of the method has been calculated on the basis of results obtained with pure I and also of those obtained with chromatographed samples of I. The dispersion of results (i.e. $3 \cdot s_{\bar{x}}$) was $\pm 4{\cdot}8\%$, the dispersion of the values of the chromatographed I was $\pm 5{\cdot}1\%$. The intensity of the light source is an important factor.

According to the recovery of I added to four samples of cabbage and to four samples of brussels sprouts, the accuracy of estimation is within the range of 12%.

DISCUSSION

The direct estimation of I without its conversion to fluorescent derivatives is difficult. I decomposes very fast under the effect of u.v. radiation.[17] It appears that I itself does not fluoresce, but is transformed rapidly into a substance or several substances, which are fluorescent (activation peak at 280 nm, fluorescence peak at 355 nm). For the present, it is not possible to utilize the decomposition of I by u.v. radiation for its estimation because of the fast-changing fluorescence intensity.

Generally there are complaints about the small difference in wave-lengths between activation values and the fluorescence maxima when indoles are directly estimated on the spectrofluorimeter. The activation peaks of the indoles in question can be found in the range of 275–295 nm and the fluorescence peaks in the range of 360–375 nm.[11,12] When measuring the fluorescence intensity yielded by the reaction of I with PDAB, it was found that the maximum values lie in a different region with a shift to the longer wave-length (i.e. the activation peaks are at 320 and 396 nm, the fluorescence peak at 490 nm). We also investigated the fluorescence peak for indolylpyruvic acid, which after reaction with PDAB also fluoresces; with the spectrophotometer CF 4 (the activation radiation was allowed for by a 330 nm filter) a value of 460 nm was found, which is different from that of I. It may be possible to use the difference of the position of the peaks of the individual fluorescing derivatives for indole identification.

Indoles which fluoresce have the general formula I $-CH_2-C\overset{O}{\underset{R}{<}}$, e.g. indolylpyruvic acid, indolylacetamide, and indolylacetic acid. Indolylacetonitrile can be saponified in a hot acidic medium into substances of the same chemical structure. In this connexion it is interesting to note the intensive fluorescence of I, which contains the group I $-CH_2-C\overset{N-R_1}{\underset{S-R_2}{<}}$. By contrast, 5-hydroxyindolylacetic acid yields only a weak fluorescence with PDAB. Furthermore, apart from I derivatives, the remaining glucosinolates do not fluoresce; this is certainly true of glucoiberin, which lacks an indole ring.

The fluorimetric method compared with the method of I estimation on the basis of the liberated SCN^- colorimetry is to a certain extent slower, requires a chromatographic separation, but it is more specific and there is no danger of interaction with other possible sources of the SCN^- ion (e.g. II, sinalbin).

EXPERIMENTAL

Chemicals and Apparatus

Spectrophotometer Spekol (Zeiss Jena) with a mercury vapour lamp HQE 40 and with an adapter for fluorimetry, spectrophotometer CF 4 (Optica Milano) with a hydrogen discharge tube and adapter for fluorimetry and the Farrand spectrofluorimeter were variously used. Reagent I for measurements on the Spekol was a 0·06% solution of PDAB in conc. HCl, the reagent II used for measurements on the spectrophotometer CF 4 was a 1% solution of PDAB in conc. HCl.

Crystalline I was isolated from plant material by a modification[10] of the method of Gmelin and Virtanen.[1] The purity of I was checked by chromatography, where it yielded a single spot in several solvent systems, by determination of the glucose, sulphate and thiocyanide content after its splitting by myrosinase and finally by u.v. spectrophotometry.[1] The preparation was 94–96 per cent pure. For the chromatography of I on paper (Whatman 1) *n*-butanol–HOAc–H_2O (4:1:2) was used. I was detected by the Procházka formaldehyde reagent (formaldehyde 40%, conc. HCl, water) (1:1:2)[16] or by the 1% solution of *p*-dimethylaminocinnamaldehyde in conc. HCl–EtOH (1:1).[7]

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[17] N. MICHAJLOVSKIJ, *Experientia* **24**, 223, (1968).