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Formation of Tryptophol Galactoside and an Unknown Tryptophol Ester in *Euglena gracilis*[1]

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ABSTRACT

The unicellular alga *Euglena gracilis* Klebs 'Z' converted exogenous indole-3-ethanol (tryptophol) to two major metabolites: tryptophol galactoside and an unknown compound, and to minor amounts of indole-3-acetic acid, tryptophol acetate, and tryptophol glucoside. The unknown was hydrolyzed to tryptophol by methanolic ammonia and should therefore be a tryptophol ester. The galactoside was identified as 2-(indol-3-yl)ethyl-β-D-galactopyranoside. This structure was established by comparison with an authentic standard involving chromatographic methods, ultraviolet and mass spectroscopy, enzymic and acid hydrolysis, and identification of the galactose in the hydrolysate. By forming tryptophol galactoside, *Euglena* differs from the higher plants examined so far, for which the corresponding glucoside is the only sugar conjugate of tryptophol detected.

In IAA biogenesis in plants, indole-3-acetaldehyde, the immediate precursor of the growth hormone, may also be reduced to tryptophol[2] (18). The reverse reaction has likewise been observed, and the set of oxidases and reductases involved appears to be the only enzyme system in IAA biogenesis in higher plants which, to some extent, responds to feedback inhibition by IAA and has thus been suggested to play a part in auxin homeostasis (3, 4, 16).

Such a regulatory mechanism would also be influenced by further metabolic reactions of tryptophol as, for example, its reversible conjugation with glucose which has been observed in many higher plants (13, 14; G. Laćan, V. Magnus, unpublished). Glucosides are common metabolites of plant growth hormones and biogenetically related compounds (5, 19). Sugar conjugates containing other saccharide moieties have less frequently been identified (8, 21). Here we report on the transformation of tryptophol to its β-D-galactopyranoside and other conjugates by the unicellular alga *Euglena gracilis*.

MATERIALS AND METHODS

Chemicals. The glycosides of tryptophol and their tetraacetates were synthesized (11; G. Laćan, V. Magnus, unpublished). Other chemicals and enzymes were used as obtained commercially. Only pyridine and acetic anhydride were purified by drying with Drierite (anhydrous $CaSO_4$) and distilled. Tetrahydrofuran was distilled in a nitrogen atmosphere over potassium and benzophenone to remove peroxides. Ethylenediamine was distilled under reduced pressure (15 mm Hg). Column chromatography was on silica gel, particle size 0.063 to 0.2 mm (Kemika, Zagreb, Yugoslavia) and on Sephadex G-15 (Pharmacia AB, Uppsala, Sweden). Silica gel GF for analytical, and silica gel HF (Kemika, Zagreb, Yugoslavia) for preparative TLC were coated on glass plates according to standard procedures. K/Na phosphate buffer (pH 7) was prepared from 11.94 g/l $Na_2HPO_4 \cdot 12\ H_2O$ and 4.54 g/l KH_2PO_4. Ethylenediamine-acetate buffers contained, per liter, glacial acetic acid (6 ml) and freshly distilled ethylenediamine: 1.15 ml for pH 4.2 and 4.65 ml for pH 7.4.

Plant Material. The strain of *Euglena gracilis* Klebs 'Z' was kindly provided by Dr. E. Marčenko. Axenic cultures were grown in a medium containing yeast extract (2 g/l), peptone (2 g/l), and sodium acetate (3.28 g/l using the anhydrous salt), the pH being adjusted to 5.9 with HCl. Culture flasks were filled to a height of about 10 cm. They were not agitated or aerated. The cultures were maintained for 2 to 3 weeks, at 20 ± 5°C, 2000 to 3000 lux of 'white' fluorescent light, and a light-to-darkness cycle of 14 to 10 h. During this time, the pH of the medium gradually changed to about 7.

Cells were harvested by centrifugation at 250*g*, at 0 to 5°C. The pellet was suspended in sterile K/Na phosphate buffer and recentrifuged; this was repeated. The weight of the final pellet was taken as a measure of the quantity of *Euglena* isolated. Yields were 5 to 7 g of cells/l culture medium.

Incubation and Fractionation of Metabolites. *Euglena* cells were suspended in tryptophol solutions in K/Na phosphate buffer and incubated, with aeration and occasional manual shaking, at room temperature (22 ± 2°C), for 5 h, in dim light (300–400 lux). Two experiments were performed using different tryptophol concentrations and different procedures for the isolation of metabolites.

Experiment 1. Nine grams of *Euglena* were incubated in 80 ml of buffer containing 0.8 mg/ml of tryptophol. The suspension was then passed through a 'French' pressure cell (cell diameter, 1 inch; pressure, 1020 atm). The homogenate was immediately mixed with methanol (500 ml) and cooled to −8°C. Eleven grams of *Euglena*, which had not been incubated with tryptophol, were processed in the same way, as a control. The extracts were evaporated to 1 to 2 ml, followed by addition of methanol (50 ml), cooling (+4°C), and removal of the precipitate formed. This was repeated, concentrating to successively smaller volumes, and adding correspondingly smaller amounts of methanol, until no further precipitation was effected by this solvent. After a final precipitation step, using benzene:methanol (5:1, v/v), the sample was concentrated (0.5 ml) and applied to a column (9 × 1 cm) of silica gel (3.8 g) packed in benzene:methanol (20:1, v/v). This solvent was used as the first eluent (30 ml—fraction 1), followed by benzene:methanol (15:1, v/v, 30 ml—fraction 2), and diethylether:methanol:water (30:10:1, v/v/v, 35 ml—fraction 3). When a mixture of authentic standards was separated by this

[1] Supported by the Research Council of the Republic of Croatia (SIZ-II).

[2] Abbreviation: tryptophol, indole-3-ethanol or 3-(2-hydroxyethyl)indole

method, tryptophol appeared in fraction 1, tryptophol glycosides and indole-3-acetic acid in fraction 3.

Experiment 2. Euglena (75 g) was incubated in 400 ml of buffer containing 0.4 mg/ml of tryptophol. After incubation, the cell suspension was not homogenized but immediately poured into a 10-fold volume of methanol and cooled (−8°C). This should inactivate glycosidases set free by cell lysis. The sample obtained was concentrated *in vacuo* to 100 ml and centrifuged. The supernatant was further evaporated to 30 ml, and this concentrate was mixed with 30 ml of acetone and left at +4°C for 2 h. The precipitate was removed by centrifugation. The supernatant (acetone plus water layers) was concentrated to 10 ml, acidified with acetic acid to pH 4.2, and cooled (+4°C, 3 h). After removal of any precipitate, by centrifugation, the supernatant was applied to a column (32 × 2.5 cm) of Sephadex G-15 equilibrated with ethylenediamine-acetate (pH 4.2). This buffer was used as the first eluent (800 ml), followed by ethylenediamine-acetate (pH 7.4, 100 ml), water (700 ml), and methanol (1000 ml). Fractions were collected in accordance with empirical experience (15), *i.e.* in succession: A (300 ml), B (150 ml), C (300 ml), D (250 ml), E (100 ml), F (500 ml), G (1000 ml). Fractions containing a buffer were lyophilized, and the residue warmed to 40°C, *in vacuo*, to sublime ethylenediamine-acetate. Other fractions were evaporated *in vacuo*. The residues were dissolved in methanol, and aliquots analyzed by TLC.

When the Sephadex column was calibrated with authentic standards, IAA was eluted in fraction E, and tryptophol galactoside in fraction C.

TLC. Indolic compounds in fractionated *Euglena* extracts were tentatively identified by TLC, using authentic standards. Solvents were: A = chloroform:methanol:acetic acid (90:10:1, v/v/v); B = chloroform:ethanol (80:20, v/v); C = 2-propanol:ethyl acetate:0.5 M H_3BO_3:NH_4OH (35:45:10:10, v/v/v/v); D = 2-propanol:hexane:water (55:30:11, v/v/v); E = chloroform:benzene (1:1, v/v). Indoles were detected by UV absorbance and by spraying with the Ehrlich reagent (10 g/l of *p*-dimethylaminobenzaldehyde in ethanol:concentrated HCl, 1:1, v/v). Quantities of metabolites were generally estimated, by order of magnitude, from spot areas on chromatograms. In some cases, more stringent identification and quantification were attempted.

GC-MS. The putative tryptophol galactoside (5 μg) isolated as a *Euglena* metabolite was dried and acetylated in a dry-box, for 10 min, at room temperature, using a mixture of 4% (w/v) dimethylaminopyridine in pyridine (50 μl) and acetic anhydride (50 μl) (10). The reagent was then evaporated and the residue dissolved in tetrahydrofuran (10 μl). The acetylation was carried out under nitrogen, a stream of nitrogen was used for evaporation and drying, with the sample temperature not exceeding 40°C. Authentic tryptophol galactoside was acetylated under the same conditions to check the performance of the derivatization.

Aliquots (1–2 μg) of the acetylated glycosides were analyzed by GC-MS using a column (180 cm × 2 mm) of 3% OV-17 on Gas Chrom Q (100–200 mesh) operated with helium at 30 ml/min as the carrier gas, and the temperature programmed from 250 to 300°C (10°C/min, hold at 300°C). The mass spectrometer was a quadrupole instrument (Hewlett-Packard 5985 a). Electron impact ionization at 70 ev was used.

Carbohydrate Analysis. An amount of the putative tryptophol galactoside from *Euglena* corresponding, by UV absorption, to 55 nmol of the tryptophol chromophore was hydrolyzed with 400 μl of trifluoroacetic acid at 100°C, for 3 h, in an evacuated sealed ampoule. Part of the hydrolysate (250 μl) was processed on a Biotronik LC 2000 carbohydrate analyzer equipped with a column (18.5 × 0.6 cm) of Durrum DA-X8-11. A routine elution program, which permitted clear separation of the common pentoses and hexoses, was used employing the following sequence of borate buffers: 0.1 M, pH 8.00; 0.2 M, pH 8.55; 0.3 M, pH 8.99; 0.4 M, pH 9.31; 0.5 M, pH 10.00. The sugar eluted was reacted with copper bicinchoninate (4,4′-dicarboxy-2,2′-bisquinoline) and the absorbance of the colored product at 570 nm was recorded (Fig. 3). Peak areas were determined by electronic integration and converted to sugar equivalents using two different standards: (a) free galactose; (b) authentic tryptophol galactoside. Before analysis, both standards were subjected to the same treatment as used for hydrolysis of the putative tryptophol galactoside from *Euglena*.

RESULTS

General. When *E. gracilis* was isolated from liquid cultures, the majority of the cells lost their flagellae. However, most of the cells, *i.e.* 80% in experiment 1 and more than 90% in experiment 2, remained viable to the end of tryptophol incubation (viability checked by microscopy).

The metabolites formed by *Euglena* were separated by two methods, the performance of which had been studied before. The silica gel column (12) affords reasonable recoveries of nonpolar metabolites, while recoveries of tryptophol glycosides are only moderate. Polar indolic compounds, like tryptophan, cannot be isolated by this method. The Sephadex procedure (15) is more suitable for the separation of glycosides and other metabolites of high and intermediate polarity. Indoles as lipophilic as tryptophol esters would have required lengthy elution with organic solvents, which was not attempted.

Tryptophol Metabolites Isolated by the Silica Gel Column. When control extracts of *Euglena* were fractionated on the silica gel column, none of the fractions contained detectable amounts (by TLC) of indolic compounds.

In the case of extracts obtained after tryptophol incubation, the nonmetabolized substrate was present in fractions 1 through 3. Four metabolites were detected; they were eluted in fractions 1 and 3.

One of the metabolites in fraction 1 co-chromatographed with *O*-acetyl tryptophol (solvent E, R_F = 0.10); the second, unidentified, indole derivative had about twice that R_F value in solvent E. Both metabolites were hydrolyzed to tryptophol (identified by TLC) on treatment with methanolic ammonia (24 h, room temperature). They must therefore be esters of tryptophol. The structure of the acyl residues requires confirmation (the putative acetate) or identification (unknown ester) by unambiguous methods. This makes quantitative estimates somewhat problematic. The amount of bound tryptophol which is responsible for the Ehrlich-positive spot obtained from the putative acetate, appeared to be around 1 to 10 μg with respect to the whole *Euglena* extract obtained. A quantity of tryptophol larger by one to two orders of magnitude was incorporated in the unknown ester. This compound is, therefore, one of the most prominent tryptophol metabolites formed by *Euglena*.

Aliquots of fraction 3 were analyzed by TLC in an acidic, a neutral, and a borate-containing alkaline solvent, as specified in Table I. In all three solvents, two Ehrlich-positive spots at the R_F values of authentic tryptophol glucoside and tryptophol galactoside were observed, in addition to unmetabolized tryptophol.

Tryptophol Metabolites Isolated by the Sephadex Column. When *Euglena* extracts obtained after tryptophol incubation were processed on the Sephadex column, most indolic material was eluted in fraction C. Further analysis of this fraction by TLC (Table I) indicated the presence of tryptophan, tryptophol galactoside, and tryptophol glucoside. Spot areas on chromatograms suggested a glucoside-to-galactoside ratio around 1:100.

The putative tryptophan in fraction C was isolated by preparative TLC (solvent C) and its identity confirmed by comparing its ^1H and ^{13}C NMR spectra with those of an authentic sample. Furthermore, fraction B contained Ehrlich-positive material hydrolyzable to tryptophan (identified by TLC) by 3 N $Ba(OH)_2$ at

100°C. Possible influence of tryptophol feeding on the levels of free and bound tryptophan was not investigated; the free amino acid is known to be present in algae (1).

Fraction A from the Sephadex column contained the bulk of plant pigments and salts, impeding further analysis. Fraction D did not contain indolic compounds, while nonmetabolized tryptophol was present in fractions E to G. An amount of IAA slightly above the detection limit of the method (0.1–1 μg with respect to the whole extract) was found in fraction E. The fact that IAA was not observed in experiment 1 (silica gel column) can be explained by the smaller amount of plant material used in that case.

Table I. *Chromatographic Separation of Tryptophol Galactoside from Compounds of Related Structure and Polarity*

The R_F values presented were obtained with authentic standards. Ratios of R_F values repeatedly determined in the same solvent system were reproducible to about 5%, while individual values were more variable. Metabolites in the respective fractions of *Euglena* extracts were therefore identified by co-chromatographing on the same TLC plate: A, an aliquot of the plant extract; B, authentic standards of the indolic compounds expected; C, aliquots of the plant extract each of which had been supplemented with one of the standards. Spot positions and intensities were then compared.

Compound	R_F Value		
	Solvent A[a]	Solvent B[a]	Solvent C[a]
Tryptophol galactoside[b]	0.05	0.11	0.38
Tryptophol glucoside[b]	0.09	0.17	0.43
Tryptophol xyloside[b]	0.19	0.31	0.54
Tryptophol arabinoside[b]	0.16	0.26	0.49
Tryptophol fucoside[b]	0.17	0.30	0.50
Tryptophan[c]	0.00	0.00	0.26
Indole-3-acetic acid[d]	0.60	0.09	0.33
Tryptophol[d]	0.62	0.60	0.94

[a] Solvents: A = chloroform:methanol:acetic acid (90:10:1, v/v/v); B = chloroform:ethanol (80:20, v/v); C = 2-propanol:ethyl acetate:0.5 M H_3BO_3:NH_4OH (35:45:10:10, v/v/v/v).

[b] β-D-Pyranosides, except for the α-L-arabinopyranoside. All of these five glycosides are cleaved by β-glucosidase from sweet-almond emulsin.

[c] Coelutes with tryptophol glucoside and tryptophol galactoside on the Sephadex column.

[d] Present (tryptophol) or suspected (IAA) in the glycoside-containing fraction from the silica gel column.

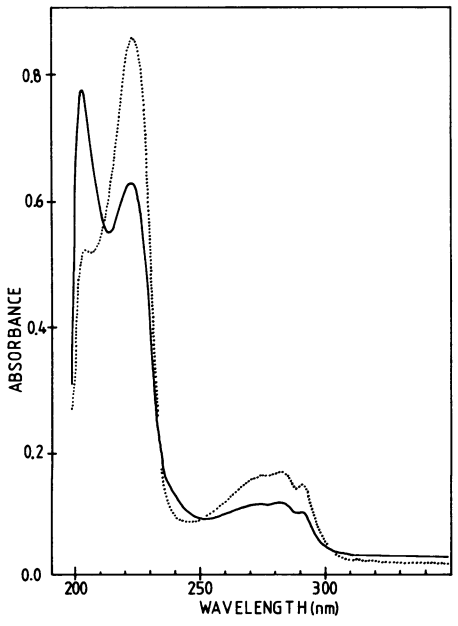

FIG. 1. Comparison of the UV spectra (in 95% ethanol) of authentic tryptophol galactoside (· · · · ·) and of the putative tryptophol galactoside isolated as a tryptophol metabolite of *E. gracilis* (————).

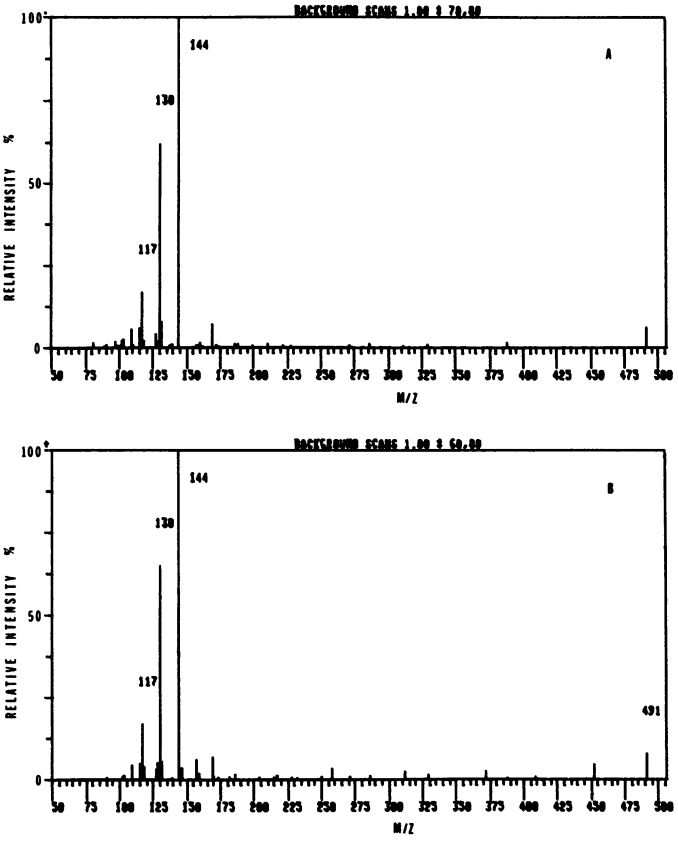

FIG. 2. Comparison of the mass spectra of (A) authentic tryptophol galactoside tetraacetate and (B) tryptophol galactoside isolated as a tryptophol metabolite of *E. gracilis* and acetylated. Diagnostically significant ions are: 491 (M^+), 144 (indole-CH_2-CH_2^+), 130 (quinolinium ion). The mass spectra were obtained by combined GC-MS. The conditions used did not in every case permit resolution of isotope clusters.

Isolation and Identification of Tryptophol Glycosides. The two tryptophol glycosides formed by *Euglena* were isolated by preparative TLC (solvent C) from the respective fractions from the silica gel or Sephadex columns.

On treatment with β-glucosidase from sweet-almond emulsin (2), both glycosides were cleaved yielding tryptophol. The enzyme is known to hydrolyze β-D-glucopyranosides, galactopyranosides, -xylopyranosides, -fucopyranosides, as well as α-L-arabinopyranosides (22). These five tryptophol glycosides were synthesized as standards, and only the glucoside and the galactoside co-chromatographed with the *Euglena* metabolites (Table I).

Only the putative galactoside was abundant enough for further analysis. It was purified by preparative TLC (solvents D and B). The UV spectrum of the sample obtained closely matched that of authentic tryptophol galactoside (Fig. 1), except for additional absorbance around 200 nm. This could be due to solvent residues from the TLC purification. Accurate extinction coefficients for authentic tryptophol galactoside could not be determined, as the compound is hygroscopic. The respective values for free tryptophol at 291 nm (ϵ = 4760), 282 nm (ϵ = 5590), and 274–275 nm (ϵ = 5240) were therefore used for quantification. This approach is justified as: a, extinction coefficients of tryptophol are not significantly (±7%) different from those of authentic tryptophol galactoside tetraacetate, which is not hygroscopic; b,

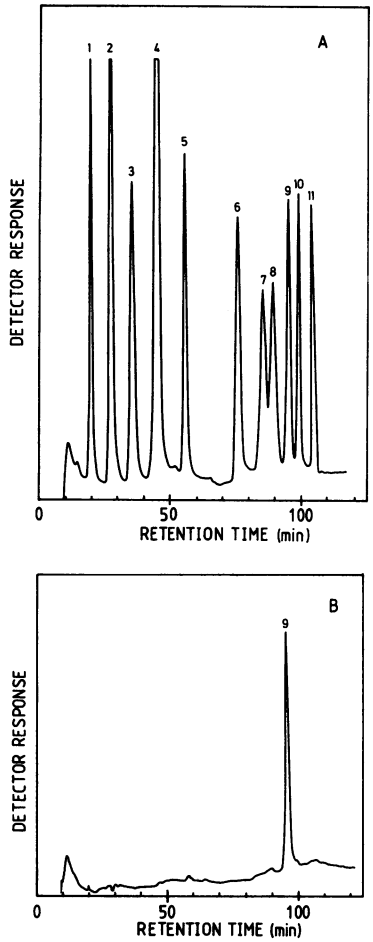

FIG. 3. Identification of galactose in the hydrolysate of a glycoside isolated as a tryptophol metabolite of *E. gracilis*, using an automatic carbohydrate analyzer. Sugars were separated as borate complexes on an anion exchanger, reacted with copper bicinchoninate, and the *A* at 570 nm was recorded. Elution patterns of the following samples are presented: (A) the standard calibration mixture of sugars used to check the performance of the separation; (B) the hydrolysate of tryptophol galactoside obtained from *Euglena*. The flow rates of both the eluent and the reagent (bicinchoninate solution) were 40 ml/h (see text for details). Sugars are (retention times in minutes in parentheses): 1 = deoxyribose (17.9), 2 = cellobiose (25.6), 3 = maltose (33.6), 4 = rhamnose (43.4), 5 = ribose (54.1), 6 = mannose (74.9), 7 = fructose (84.5), 8 = arabinose (88.6), 9 = galactose (94.6 for A, 94.8 for B), 10 = xylose (98.4), 11 = glucose (103.7).

absorbance values at 291, 282, and 274–275 nm for tryptophol galactoside are in the same proportion as the extinction coefficients for free tryptophol at the respective wavelengths.

The quantity of putative tryptophol galactoside obtained in experiment 2 was thus estimated to be 91 nmol or 29 μg (mol wt 323—quantity not corrected for losses during isolation).

Aliquots of (a) authentic peracetylated tryptophol galactoside, (b) the putative *Euglena* galactoside acetylated as described, and (c) synthetic tryptophol galactoside acetylated under the same conditions were compared by GC-MS. Retention times for all three samples were 13.5 ± 0.5 min. Their mass spectra (Fig. 2) were also identical within the limits of experimental error. This proves that the *Euglena* metabolite examined is a hexoside of tryptophol, most likely the galactoside. The mass spectrum of its tetraacetate is, however, almost indistinguishable from that of the corresponding acetylated glucoside (11). Additional evidence for the identity of the sugar moiety in the *Euglena* metabolite was therefore obtained.

The tryptophol content in an aliquot of the metabolite was determined from its UV spectrum. The sample was then hydrolyzed and the hydrolysate processed on an automatic carbohydrate analyzer. Galactose was the only sugar detected (Fig. 3). The amount of galactose bound to 34 nmol of tryptophol was 46 nmol, if calculations were based on a standard of free galactose, or 38 nmol, if authentic tryptophol galactoside was used as the standard. The latter value is considered more accurate, as the indolic moiety of tryptophol galactoside is likely to give, on acid hydrolysis, reddish decomposition products which may absorb at the same wavelength (570 nm) as the colored complex formed from galactose and copper bicinchoninate. It can therefore be concluded that the *Euglena* galactoside contains equimolar amounts of tryptophol and galactose, and no other carbohydrates.

DISCUSSION

The two preponderant tryptophol metabolites in *Euglena* were tryptophol galactoside and an unknown tryptophol ester; they were formed in amounts comparable by order of magnitude. Preliminary spectroscopic data on the unknown ester indicate a complex acyl residue, which requires further study. The galactoside was identified by: (a) co-chromatography with an authentic sample, (b) its UV spectrum which indicated an indole ring unsubstituted except for an aliphatic side chain in the 3-position; (c) enzymic hydrolysis which permitted chromatographic identification of the aglycone and established the sugar residue to be a β-D-pyranose; (d) GC-MS which gave the fragmentation pattern expected for a hexoside of tryptophol; and (e) acid hydrolysis and identification of the hexose as galactose in a quantity consistent with a sugar-to-aglycone ratio of 1:1. The metabolite is therefore to be assigned the systematic name 2-(indol-3-yl)ethyl-β-Dgalactopyranoside.

Minor metabolites were not present in quantities permitting further purification and identification. The *O*-acetate ester (6, 9) and IAA (17, 20) have been observed as tryptophol metabolites in plants. The glucoside was originally isolated from pea seedlings fed with tryptophol, using purification methods very similar to those applied here, and identified by mass spectroscopy combined with enzymic cleavage and chromatographic identification of the sugar fragment as glucose. This supports our identification of the glucoside as one of the metabolites of tryptophol in *Euglena*.

Thus, while the glucoside is a major metabolite of tryptophol in many higher plants (13, 14; G. Laćan, V. Magnus, unpublished), the respective galactoside is much more prominent in *Euglena*. If this should be a general difference between algae and vascular plants, it may be of taxonomic significance.

Concerning the physiological relevance of this work, IAA tends to be considered a growth hormone in algae, although evidence has often been controversial (1). This acid is reported to inhibit the growth of *E. gracilis*, at a concentration of 10 mg/l, while smaller concentrations have no effect (7). Tryptophol metabolism, which is connected to the biogenesis of IAA in higher plants, may thus be physiologically significant in *Euglena* as well. Tryptophol concentrations above hormonal levels were used in this work to obtain one of its principal metabolites, the galactoside, in quantities permitting unequivocal identification. This information should facilitate future work using substrate levels closer to plausible endogenous concentrations.

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