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OCCURRENCE OF NORSPERMINE IN EUGLENA GRACILIS

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SUMMARY. The tetraamine occurring in Euglena gracilis, previously believed to be spermine, is shown to be norspermine[N, N'-bis (3-aminopropyl)-1,3-diaminopropane]. Proof of identity was established by HPLC, HPCC and mass spectrometric investigations.

The polyamines putrescine, spermidine and spermine are widely distributed in biological materials and have been linked mainly to the biosynthesis of proteins and nucleic acids. Their physiological functions have been recently reviewed (1).

The polyamine content of Euglena gracilis has been reported previously (2,3), and the only tetraamine found in this alga was thought to be spermine [$H_2N\ (CH_2)_3NH\ (CH_2)_4NH\ (CH_2)_3NH_2$].

We now present results obtained with high-performance liquid chromatography (HPLC), high-performance cation-exchange chromatography (HPCC) and mass spectrometry which has led to the identification of norspermine (N, N'-bis (3-aminopropyl)-1,3-diaminopropane)[$=H_2N\ (CH_2)_3NH\ (CH_2)_3NH\ (CH_2)_3NH_2$] as the major tetraamine in Euglena gracilis.

MATERIALS AND METHODS

Chemicals - All chemicals were the purest grade available and purchased from Merck, Darmstadt, except dansyl chloride which was obtained from Fluka.

Algal samples - Cells of Euglena gracilis (strain Z) were grown in axenic cultures at 25°C either in the dark (4) or under autotrophic conditions (5).

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Cells of the same strain were also grown in mass cultures according to Stengel (6), harvested by centrifugation, lyophilyzed and extracted with 0.2M $HClO_4$ (90°C, 15 min). The extracts were used for analysis.

Synthesis of norspermine - The synthesis was performed using a method similar to that described for hydroxypolyamines (7).

0.05 mol of 1,3-dibromopropane was added to 0.6 mol of 1,3-diaminopropane and maintained at 80°C for 3 hours. Excess diaminopropane and volatile reaction products were then removed by distillation under reduced pressure (12 mm Hg). The residue was purified by dissolving in water and applying it to a column (25 x 2.5 cm) filled with a weakly acidic cation-exchange resin (CG 50-II, Serva, H^+-form). The amines were eluted with 0.5M HCl, and an aliquot of each fraction spotted on alumina 60 TLC plates. After developing the plate in n-butanol-acetic acid-water (4:1:1), the amines were detected by spraying with ninhydrin reagent.

The purest fraction contained norspermine tetrahydrochloride (1 g) of >99 % purity. Analysis of the product by ^1H-(δ = 2.3 - 2.7, m, 6H; δ = 3.4 - 3.8, m, 12H) and ^{13}C-NMR spectroscopy (δ = 24.0, 25.0, 37.9, 45.9 and 46.1) was in agreement with the structure of norspermine.

Calcul. : C 32.35 %; H 8.45 %; N 16.80 %; Cl^- 42.44 %.
found : C 32.22 %; H 8.31 %; N 16.78 %; CL^- 42.33 %.

HPCC of polyamines - Extracts of Euglena were analysed in 10 - 80 µl aliquots using a Durrum D-500 amino-acid analyser (Durrum Instrument Corp.) according to a procedure previously described (8,9). Retention times were: spermidine - 39 min, norspermine - 45 min, spermine - 50 min.

Preparation of dansyl derivatives - The method of Seiler and Wiechmann (10) was slightly modified. To 0.2 ml algal extract in 0.2M $HClO_4$ (50 mg algae in 1 ml extractant) 0.3 ml 10 % Na_2CO_3 and 0.8 ml of a solution of dansyl chloride in acetone (7.5 mg/ml) were added. After reacting for 20 min at 60°C, the excess dansyl chloride was removed by addition of 0.1 ml of a 10 % solution of proline and subsequent heating (60°C, 10 min). The mixture was then cooled to 5 - 10°C and extracted with 100 µl benzene. After separation of the layers, 5 - 10 µlof the supernatant were taken for analysis. Preparation of dansyl derivatives was performed in the dark. For quantitative determinations a known amount of 1,8-diamino-octane (as internal standard) was added to the algal sample prior to extraction.

HPLC for dansyl derivatives - A separation of the dansyl derivatives was effected using a Waters (GmbH) Liquid Chromatograph equipped with M 6000 and M 6000 A pumps, a M 660 programmer and a µ-Bondapak C-18 reverse-phase column (30 x 0.4 cm, 10 µ particles). Detection was achieved with a Fluoropal (Winopal, Hannover) fluorimeter equipped with a 7 µl flow cell. The excitation wavelenghth was λ = 333 nm, while emitted light was passed through a cut-off filter of λ = 408 nm. A linear gradient of 40 - 80 % acetonitrile/water at 40°C with a flow rate of 1 ml/min was used to elute the dansylated products. Retention times for the dansyl derivatives were: spermidine - 48.0 min, norspermine - 60.8 min, spermine - 61.7 min.

Mass spectrometry - A varian Mat mass spectrometer CH 7 with a direct inlet system and data processing by Spectrosystem 100 MS was used with electron energy 70 eV, trap current 100 µA, and ion source temperature 400°C.

RESULTS

Analysis of Euglena gracilis extracts with HPCC and of the dansyl derivatives with HPLC showed the existence of a distinct component which

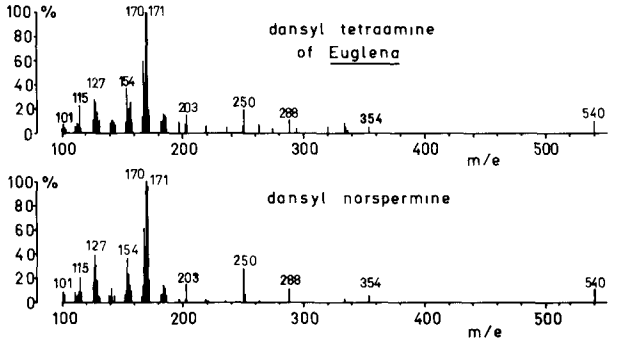

Fig 1 Mass spectrum of the dansylated tretraamine of Euglena and of dansyl norspermine

is eluted slightly earlier than spermine but has the same retention time as synthesized norspermine. Spermine was present only in traces. The addition of spermine to extracts of Euglena yielded a new peak in the chromatogram which again indicated that the major tetraamine in Euglena is different from spermine. The dansyl derivative of the tetraamine of Euglena, obtained from a dansylated algal extract by semi-preparative HPLC under analytical conditions, had a mass spectrum which was in good agreement with the mass spectrum of standard dansyl norspermine as shown in fig. 1. Due to the instability of the highly dansylated molecule both spectra do not show the molecular ion (m/e = 1120).

From these results it can be concluded that the tetraamine of Euglena gracilis is identical with norspermine. The concentration of norspermine in cells from a mass culture was found to be 4.5 μmoles/g (dry wt.).

DISCUSSION

The occurrence of norspermine in Euglena is a further example of the presence of "unusual" polyamines in algae. Since Euglena also contains norspermidine and homospermidine, it is an exceptionally good source of these homologues which seem to be less prevalent in nature than spermidine and spermine themselves. To our knowledge norspermine has been found only

in thermophilic bacteria (11,12) and in the white shrimp Pennaeus setiferus (13). It is likely that application of analytical methods with sufficient resolution might also detect more complex patterns of polyamines in other organisms.

Biosynthetic pathways for norspermine as well as for other unusual polyamines (e.g. norspermidine and sym-homospermidine) have been proposed (12,14). The presence of these polyamines and the virtual absence of spermine in some organisms raises the question of their physiological functions and of the control mechanism involved in their biosynthesis.

The presence of norspermine in Euglena indicates that its metabolic function is not only related to thermophily. Therefore, the trivial name of norspermine for 1,11-diamino-4,8-diazaundecane(=N,N' -bis (3-aminopropyl) -1,3-diaminopropane) , indicating its relationship to spermine, seems preferable to that of thermine (11).

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