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BIOSYNTHESIS OF POLYAMINES IN *EUGLENA GRACILIS*

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Abstract—In *Euglena gracilis* Z the biosynthesis of spermidine and spermine closely resembles the pathways occurring in mammalian tissues and in most microorganisms. L-Ornithine and not L-arginine, as is the case in most plants, is the main precursor of putrescine, and *S*-adenosylmethionine donates the propylamino moiety for the biosynthesis of spermidine and spermine. Cell-free extracts of *Euglena* synthesized *sym*-norspermidine and *sym*-norspermine from 1,3-diaminopropane and labelled *S*-adenosylmenthionine. The synthases for the biosynthesis of these two polyamines have a pH optimum of 7.6, like that of spermidine and spermine synthases. Ion exchange chromatography showed two peaks corresponding to the retention times of 2,4-diaminobutyric acid and 1,3-diaminopropane, lower homologues of ornithine and putrescine, respectively. Experiments with DL-2,4-diaminobutyric acid-[4-^{14}C] did not result in significant incorporation of the label into 1,3-diaminopropane.

INTRODUCTION

Studies concerning the polyamines in plants and microorganisms have led to the identification of a large number of unusual polyamines, e.g. *sym*-norspermidine (Nor SPD), *sym*-norspermine (Nor SPN), *sym*-homospermidine and aminopropyl cadaverine [1–9], in addition to the widely distributed common polyamines, spermidine, spermine and putrescine [9–15]. The presence of Nor SPD and Nor SPN was claimed to be peculiar to thermophilic microorganisms [1, 2, 16]. However, we have recently shown [6] that *Euglena gracilis* Z has a polyamine composition similar to that of thermophilic microorganisms; moreover, Nor SPN is its major polyamine component. We found it particularly interesting, therefore, to study the biosynthetic pathways of these polyamines in *E. gracilis* in order to compare them with those of the thermophilic microorganism *Caldariella acidophila* [16]. The possibility of the presence of a 2,4-diaminobutyric acid decarboxylase leading to the biogenesis of 1,3-diaminopropane (DAP), which is the hypothetical intermediate for the biosynthesis of Nor SPD and Nor SPN, was also investigated.

RESULTS AND DISCUSSION

Incorporation of labelled precursors

Cell cultures or homogenates of *E. gracilis* were incubated with methionine-[2-^{14}C], ornithine-[5-^{14}C], arginine-[5-^{14}C] and diaminobutyric acid-[4-^{14}C]. Except for *in vivo* incubation with DL-methionine-[2-^{14}C] a very poor incorporation of the precursors into polyamines resulted. Subsequent studies of the influence of the composition or the pH of the medium or the buffers did not improve our earlier results.

About 9% of the radioactivity of methionine incubated was incorporated into the cells of *E. gracilis*. The incorporated activity of methionine was mainly recovered in Nor SPD (7%), spermidine (6%) and Nor SPN (77.4%). This indicates that the propylamino moiety for the biosynthesis of spermidine, Nor SPD and Nor SPN is donated by methionine. The differences observed in the percentage of radioactivity incorporated into these 3 polyamines possibly result from their diversified turnover rates and/or from their cellular compartmentation.

In vitro *incorporation of labelled precursors*

The previous observations that polyamines inhibit their own biosynthesis [17–19] led us to consider that the poor incorporation of the different precursors in our *in vitro* experiments could be due to the inhibitory effect of the considerable amounts of polyamines present in *E. gracilis* [5, 6]. Experiments were designed therefore to eliminate most of the endogenous polyamine content from the *E. gracilis* homogenates using a Sephadex G-50 (Medium) column. The homogenate, virtually free of polyamines, was then incubated in seperate experiments with different ^{14}C-marked polyamines precursors. The results were greatly improved and a very high level of incorporation of labelled precursors was obtained.

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Biosynthesis of purtrescine, spermidine and spermine

Table 1 illustrates the distribution of radioactivity from the various precursors into the different polyamines detected in *E. gracilis*. Although putrescine is formed by ornithine decarboxylase and also by arginine decarboxylase via agmatine, it is noteworthy that ornithine, and not arginine, as is the case in most of the plants [20, 21] is the main precursor. When extracts of *E. gracilis* were incubated with ornithine, we found besides putrescine, radioactivity in hydroxy proline, glutamate and citrulline. The maximum amount of putrescine was obtained at pH 5.5 from arginine and at pH 7.2 from ornithine.

Arginine also gave rise to small amounts of ornithine probably as a result of arginase activity. Small amounts of hydroxy proline, glutamate and citrulline were also detected.

An appreciable amount of radioactive putrescine was also obtained from citrulline-[5-^{14}C]. But our isotopic dilution experiments with radioactive citrulline in the presence of unlabelled ornithine considerably diluted the specific radioactivity of the putrescine peak, thereby demonstrating that citrulline is first converted to ornithine which in turn gives rise to putrescine.

The results presented here indicate that the biosynthetic pathways of spermidine and spermine closely resembled that demonstrated in microorganisms [11] and mammalian tissues [15, 22–24] in which putrescine provides the 1,4-diaminobutane moiety and decarboxylated *S*-adenosylmethionine (De SAM) donates the propylamino group.

Biogenesis of DAP and biosynthesis of Nor SPD and Nor SPN

De Rosa *et al.* [16] have reported that in *Caldariella acidophila*, DAP is the free intermediate in the biosynthesis of Nor SPD and Nor SPN, and the De SAM provides the propylamino moiety. Based on the results of their experiments with double-labelled spermidine (one with ^{14}C in the tetramethylene moiety and the other with ^{3}H in the propylamino moiety) they proposed that spermidine could play a role of precursor of DAP. It has also been reported that DAP is a catabolic oxidation product of spermidine or spermine [9, 16].

Analysis on three HPLC ion exchange systems of cell extracts from *E. gracilis* revealed the presence of small amounts of DAP and 2,4-diaminobutyric acid (DABA). These results led us to test the possibility that DAP could result from the enzymatic decarboxylation of DABA by a pathway analogous to that for the biogenesis of 1,4-diaminobutane or 1,5-diaminopentane [25, 26]. Moreover, since the unusual polyamines Nor SPD and Nor SPN and their hypothetical precursor DAP have one $-CH_2$ group less than their corresponding polyamines, spermine and their precursor putrescine, and also as compared to ornithine, DABA has one $-CH_2$ group less, it was therefore quite likely that DABA could serve as the initial substrate in the biosynthetic pathways of Nor SPD and Nor SPN. However, when homogenates of *E. gracilis*, free from endogenous polyamine content, were incubated with DABA-[4-^{14}C] in absence or presence of pyridoxal phosphate and at different pH (from 4.5 to 9) no significant amount of incorporation of the label was found in DAP. Furthermore, no significant incorporation was obtained either in Nor SPD or in Nor SPN when homogenates in *E. gracilis* were incubated with labelled DABA in the presence of unlabelled SAM. However, incubation in the presence of unlabelled DAP and SAM-[3,4-^{14}C] resulted in the synthesis of labelled Nor SPD and Nor SPN. These results demonstrate that DAP serves as the free intermediate and DE SAM denotes the propylamine moiety for biosynthesis of Nor SPD and Nor SPN.

The effect of pH on the formation of Nor SPD from DAP was studied. The aminopropyl transferase has a pH optimum of 7.6 in phosphate buffer and seems to have a preference for putrescine. Addition of 0.1 μm putrescine in the incubation mixture resulted in an inhibition by 7% of the synthesis of Nor SPD from DAP, whereas 1 μM putrescine inhibited Nor SPD synthesis by about 80%. At the same time, spermidine and spermine biosynthesis was increased.

Fig. 1 indicates the effect of the concentration of

Table. 1. Distribution of radioactivity from labelled precursors into common polyamines (putrescine, spermidine and spermine) of *Euglena gracillis Z.*

	Total count (dpm)	Putrescine		Spermidine		Spermine		Citrulline		Ornithine				Agmatine		Glutamate		Hydroxyproline	
		dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%
DL-Ornithine-[5-^{14}C]	126 000	48 000	38.2					3300	2.6	48 600	38.6	400	0.3			8200	6.5	8200	6.5
DL-Arginine-[5-^{14}C]	66 100	1600	2.4					2700	4.2	1300	2.0	51 500	77.9	500	0.8	400	0.7	100	0.2
DL-Citrulline-[5-^{14}C]	124 400	9800	7.9							3300	2.7								
DL-Citrulline-[5-^{14}C](10 μM) + ornithine	128 100	9500	7.7							3400	2.7								
DL-Citrulline-[5-^{14}C] + Ornithine (100 μM)	113 900	5700	5.0					81 900	71.9	8800	7.7					2850	2.5	1250	1.1
DL-Ornithine-[5-^{14}C] + SAM (1 mM)	174 500	12 200	7.0	29 700	17.0	4600	2.7	5100	2.9	80 400	46.1					20.200	11.6	10 900	6.2
Putrescine-[1,4-^{14}C] + SAM (1 mM)	95 200	1300	1.3	71 300	74.9	18 500	19.4												

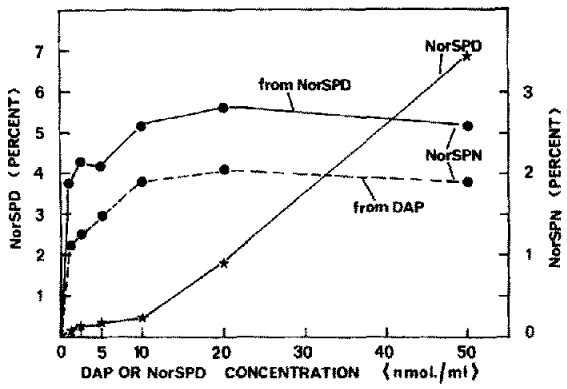

Fig. 1. Effect of concentration of 1,3-diaminopropane and Nor SPD on the biosynthesis of *sym*-norspermidine (Nor SPD) and *sym*-norspermine (Nor SPN). The concentration of the labelled *S*-adenosyl-L-methionine remained constant. Experiments were carried out at pH 7.6.

DAP and Nor SPD on the biosynthesis of Nor SPD and Nor SPN from DAP and Nor SPD. As shown, the formation of Nor SPD from DAP increases with the rise in concentration of DAP. In contrast, the rate of formation of Nor SPN either from DAP or Nor SPN increases very rapidly in the beginning but thereafter reaches almost a plateau, the concentration of DAP or Nor SPD used has practically no effect on the formation of Nor SPN. Nor SPD and Nor SPN were claimed to be peculiar and necessary components of thermophilic microorganisms [1, 2, 16]. The presence of these unusual polyamines in the white shrimp *Pennaeus setiferus* [3] and *Euglena* [5, 6] suggests that the metabolic functions of these polyamines are not restricted to only thermophily although their metabolic pathways appear to be quite similar to those in thermophilic bacteria. Thus the precise role of these polyamines still remains to be established.

EXPERIMENTAL

Cultures. Euglena gracilis Z cells were grown at 27° in inorganic medium supplemented with 33 nM DL-lactate (pH 3.5) under continuous light (1500 lx, Phillips Light Day) with generation times of 12 hr [6, 27].

Chemicals. DL-Ornithine-[5-^{14}C] HCl (11.6 mCi/mmol), DL-arginine-[5-^{14}C] HCl (12.3 mCi/mmol), DL-citrulline-[5-^{14}C] (13.5 mCi/mmol), *S*-adenosyl-L-methionine-[3-^{14}C] (13.5 mCi/mmol) and DL-diaminobutyric acid-[4-^{14}C] HCl (56 mCi/mmol) were purchased from the Commissariat à l'Energie Atomique, Saclay. DL-Methionine-[2-^{14}C] (11 mCi/mmol) and putrescine-[1,4-^{14}C] HCl (72.6 mCi/mmol) were purchased from New England Nuclear. Sephadex G-50 (medium) was obtained from Pharmacia. All other chemicals were of the highest purity grade available from standard commercial sources or as indicated earlier [6].

Experiments in vivo. *E. gracilis* culture (*ca* 70 ml) was incubated separately in continuous light with 10 μCi of the appropriate precursor for 48 hr. The culture was then centrifuged for 5 min at 2000 g and the cells were washed once with fresh medium and recentrifuged. The pellet was then extracted with 1 ml of 5% TCA in 0.05 M HCl at 4°. After homogenization, followed by centrifugation, the pellet was extracted again in the same way and recentrifuged. The supernatants were pooled and a 100 μl aliquot was used for analysis.

Experiments in vitro. All manipulations were carried out at 4° unless otherwise stated. About 800 ml of an *E. gracilis* culture grown to the exponential phase was centrifuged for 5 min at 2000 g and the pellet was suspended in 1–3 ml of the assay buffer. The composition of the assay buffer for the biosynthesis of putrescine varied according to the precursor as follows: (a) from DL-ornithine-[5-^{14}C]: 0.04 M Na_2HPO_4, 5 mM KH_2PO_4, 5 mM glucose and 1 mM EDTA (pH 7.2); (b) from DL-arginine-[5-^{14}C]: 0.09 M Na_2HPO_4, 0.01 M KH_2PO_4, 5 mM glucose and 1 mM EDTA (pH 5.5); (c) from DL-citrulline-[5-^{14}C]: 0.09 M Na_2HPO_4, 0.01 M KH_2PO_4, 5 mM glucose and 1 mM EDTA (pH 7.0). For the biosynthesis of spermine and spermidine the composition of the assay buffer was: 0.04 M Na_2HPO_4, 5 mM KH_2PO_4, 5 mM glucose and 1 mM EDTA (pH 7.6). The suspension was sonicated ×3 for 20 sec and centrifuged for 5 min at 2000 g. The supernatant was passed through a Sephadex G-50 (25×1.5 cm) column previously equilibrated with the corresponding assay buffer. Void vol. (30 ml) was determined by blue dextran. After elimination of the void vol. *ca* 30 ml of the eluate was collected. A 50 μl aliquot of the extract before and after passage through the Sephadex column, after deproteinization with 10 μl of 80% TCA, was analysed for its polyamine content. After addition of pyridoxal phosphate (50 μM) and dithiothreitol (5 mM), the extract was divided into 2 ml fractions into different 10 ml flasks and incubated with different labelled precursors for 3 hr at 37°. Unlabelled SAM (1 mM) and putrescine-[1,4-^{14}C] and DL-ornithine-[5-^{14}C] were added to the reaction mixture before incubation in case of biosynthesis of spermidine and spermine. The reaction was stopped by addition of 200 μl of 80% TCA. After centrifugation a 100 μl aliquot was analysed as described.

Polyamines and amino acid analysis and radioactive measurements. Polyamines were analysed with an amino acid analyser by the method described in ref. [6]. A fraction collector was coupled to the exit of the fluorimeter and 750 μl fractions were collected. Bray's soln [28] (10 ml) was added and the radioactivity was measured by scintillation. Quantification of the amines was carried out with an integrator ICAP 10 (LTT, Saint-Honorine) coupled to the fluorimeter. The chromatographic conditions and preparation of the eluting buffers and samples were as described earlier [29]. DABA was also analysed using: (a) a column (30×0.4 cm) of the Hamilton HC-x 7.00 resin (Touzart et Matignon, France) in the Li^+ form. 0.6 N (in Li^+) Li citrate buffer of pH 6.45 was employed at a flow rate of 30 ml/hr. During the first 4 min the column was maintained at 66° and thereafter at 78°; (b) a column (54×0.9 cm) of M-72 (Na^+ form) resin (Beckman Instruments, France). Two Na citrate buffers of 0.2 and 0.8 N (in Na^+) with pH of 3.15 and 4.00, respectively were employed at a flow rate of 70 ml/hr. During the first 70 min the first buffer is used and thereafter the second. Column temp. was maintained at 54°.

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