PURINE BIOSYNTHESIS: ENZYMATIC FORMATION OF RIBOSYLAMINE-5-PHOSPHATE FROM RIBOSE-5-PHOSPHATE AND AMMONIA.

M.-L. Le Gal, Y. Le Gal, J. Roche and J. Hedegaard.

Laboratoire de Biochimie Générale et Comparée,
Collège de France, Paris 5, France.

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The initial step in purine nucleotide synthesis de novo has been established to be the formation of ribosylamine-5-phosphate (PRA) from 5-phosphoribosyl-1-pyrophosphate (PRPP) and glutamine (Goldthwait, 1956) catalyzed by the enzyme, ribosylamine-5-phosphate:pyrophosphate phosphoribosyltransferase (E.C. 2.4.2.14), whose presence in biological systems appears to be general (Sartorelli and LePage, 1958; Tomisek et al., 1956). The specific inhibition of this enzyme by adenine and guanine nucleotides emphasizes its role in the regulation of purine synthesis (Wyngarden and Ashton, 1959; Nierlich and Magasanik, 1965). The formation of ribosylamine-5-phosphate by a second pathway directly from ribose-5-phosphate and ammonia has been shown to be non-enzymatic (Nierlich and Magasanik, 1961 and 1965). The possibility that this last reaction can also proceed enzymaticly has however been suggested from in vivo (Henderson, 1963; Henderson and Khoo, 1965) and in vitro studies (Herscovics and Johnstone, 1964) with Ehrlich ascites tumor cells and wheat germ preparations (Kapoor and Waygood, 1962) as well as in Escherichia coli in vivo (Le Gal and Hedegaard, 1967).

In this paper we present evidence for the direct enzymatic synthesis of ribosylamine-5-phosphate from ribose-5-phosphate and ammonia in cell-free extracts of Escherichia coli B.

Material and Methods.

E. coli B (No. 54.125) was grown overnight on a rotary shaker at 30° C in a mineral medium (Spizizen et al., 1951)

containing 0.4 % glucose. The cells were harvested by centrifugation at 0-4° C and washed twice with cold phosphate buffer (0.01 M, pH 7.2). Cell-free extracts were obtained after sonic disintegration of the cells in the same buffer containing 0.5 x 10^{-3} M glutathione. Ultrasonic treatment for 3 min.(MSE 60 W, 20 Kc/s) results in an approximately 30 % disruption of the cells. Extended sonication provokes more complete disintegration but leads to an inactivation of the enzymatic activity. The sonicate is centrifuged 20 min. at 37.000 x g to give a crude extract with approximately 20 mg/ml of protein.

The formation of ribosylamine-5-phosphate was measured by the 3-step incubation technique of Nierlich and Magasanik (1965) modified in respect to the addition of glycine at the first step. The crude extract (0.1 ml) was incubated at 37° C with 5µmoles of ribose-5-phosphate, 10 µmoles of NH_4^+, 0.2 µmoles of ATP, 2 µmoles of Mg^{2+}, 5 µmoles of glycine, 1 µmole of glutathione and 20 µmoles of Tris buffer in a total volume of 0.5 ml (final pH 8). The reaction is allowed to proceed for 10 min. The incubation mixture was adjusted to pH 7.2 by addition of 20 µmoles of Tris-maleate buffer, 0.2 µmoles of ATP, 2 µmoles of Mg^{2+} and 2.7 mg (in 0.1 ml) of a partially purified phosphoribosyl glycinamide synthetase (E.C. 6.3.1.3)(Flaks and Lukens, 1963) in a total volume of 0.69 ml. After further incubation for 10 min. the reaction was brought to a stop by addition of 15 µmoles of EDTA. The third step: the interaction between the synthesized glycinamide ribotide and IMP with the consecutive formation of 5-amino-4-imidazolecarboxamide ribotide (AICA ribotide) was then initiated by addition to the incubation mixture of 2.5 µmoles of IMP and 5 mg of protein (in 0.1 ml) of the 15-30 % ethanol fraction of a chicken liver extract containing the glycinamide ribotide:IMP transformylase (Flaks and Lukens, 1963). After 30 min. of incubation at 37° C the overall reaction was stopped by precipitation of the protein with 0.1 ml of 15 % TCA in 1 N HCl and the formation of the AICA ribotide was measured by the method of Bratton and Marshall (1939). Under these adopted experimental conditions the amount of AICA ribotide formed is a measure of the ribosylamine-5-phosphate (PRA) synthesis in the first reaction step.

Ribose-5-phosphate, PRPP, carbamylphosphate and ATP were purchased from Sigma; IMP, glutamine and glutathione are Cal-

biochem. products. Azaserine and DON (6-diazo-5-oxo-L-norleucine) are gifts from Parke, Davis and Co. (Michigan). Methylthioinosine was supplied by Cancer Chemotherapy National Service Center, NIH (Bethesda, MD.) and psicofuranine by The Upjohn Co.(Michigan). Methionine sulfoximine is a Sigma product.

Results and Discussion.

The results given in Fig. 1 demonstrate that ribosylamine-5-phosphate (PRA) is synthesized from ammonia and ribose-5-phosphate in crude extracts of E. coli B. The formation of PRA in the absence of protein is a measure for the non-enzymatic reaction under the experimental conditions. Increasing amounts of boiled enzyme do not significantly enhance the formation of PRA (Fig. 1). Furthermore, gel-filtration of the crude extract on Sephadex G-25 did not cause any appreciable loss of the overall

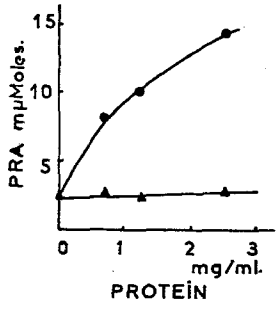

Fig. 1. Formation of PRA from ribose-5-phosphate and ammonia in the presence of crude (●) or boiled (▲) extract of E. coli B. Experimental conditions are given in detail in the text. Result at zero protein concentration is a measure of the non-enzymatic PRA synthesis.

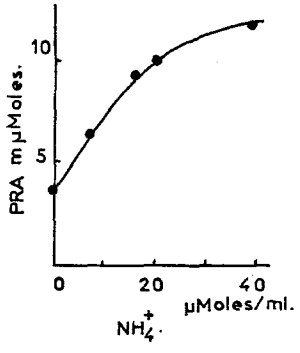

Fig. 2. PRA synthesis at increasing ammonia concentrations. Experimental conditions as in Fig. 1.

activity. Thus, these last two results eliminate the possibility that the stimulation of the glycinamide ribotide synthesis is due to a non-enzymatic activation of the PRA formation by glycine (Nierlich and Magasanik, 1965) or glutamine.

The direct enzymatic synthesis of PRA from ribose-5-phosphate and ammonia is further emphasized by the results given in Fig. 2 showing an optimal activity of the enzyme at an initial ammonia concentration of 25-30 μmoles/ml. Results on the nitrogen donor specificity for the PRA formation are indicated in Table 1. Glutamine, ammonia and carbamylphosphate are all highly potent donors in the presence of ribose-5-phosphate while asparagine is practically inactive. Under these same conditions, the lack of inhibition on the PRA synthesis by the glutamine synthetase inhibitor, methionine sulfoximine, indicates that the nitrogen donor activity of ammonia is not due to an intermediary formation of glutamine (Table 1). The decrease of activity observed after 12-16 hours of dialysis of the crude extract may result from either a loss of cofactor or the general instability of the enzyme system. On the contrary, when PRA is formed at the expence of PRPP, only glutamine and, to a lower

Table 1. Activity of various nitrogen donors in PRA synthesis from ribose-5-phosphate or PRPP.

Nitrogen donor (+)	mμmoles of PRA formed from		
	Ribose-5-P		PRPP
	Crude extract	Crude extract dialyzed	
None	3.0	1.5	1.4
Ammonia	7.0	—	3.5
Asparagine	4.0	—	1.5
Carbamyl-P	6.7	—	7.0
Glutamine	8.5	—	9.0
Ammonia	9.0	3.2	—
Ammonia + MSOI	8.7	3.2	—

(+) All nitrogen donor concentrations are 20 μmoles/ml as indicated for ammonia in the text. Methionine sulfoximine (MSOI) is tested at 1 μmole/ml. Concentrations of ribose-5-phosphate and PRPP are 10 μmoles/ml.

extent, carbamylphosphate react as nitrogen donors while ammonia and asparagine are inactive (Table 1). These results on PRA synthesis from PRPP and glutamine are in agreement with those of Nierlich and Magasanik (1961) and Kapoor and Waygood (1962). The low but significant PRA formation from PRPP and ammonia may be explained by the direct reaction between the latter and ribose-5-phosphate derived from hydrolysis of PRPP.

When the PRA synthesis in the presence of ribose-5-phosphate and ammonia is optimum, the reaction can be partially inhibited by DON (Table 2) while psicofuranine and azaserine are inactive. PRA formation at the expence of PRPP is highly inactivated by DON while neither psicofuranine nor azaserine have any effect.

Table 2. Inhibition of PRA synthesis.

Inhibitor (+)	% Inhibition of PRA synthesis at the expence of (++)	
	Ribose-5-P and ammonia.	PRPP and glutamine.
None	0	0
Azaserine	0	6
DON	19	53
Psicofuranine	0	0
Methylthioinosine	30	33

(+) All inhibitors are tested at a final concentration of 1 µmole/ml.
(++) Experimental conditions same as in Table 1.

Results on the inhibition by GMP and AMP of the PRA formation are given in Fig. 3. Both purine nucleotides exert a feed-back control on the PRA synthesis from ribose-5-phosphate and ammonia in the cell-free extract of E. coli B. Maximum effect (80-100 % inhibition) is obtained at approximately 10^{-3} M concentrations of AMP and GMP. The IMP analog, methylthioinosine, at this same concentration, inhibits the overall reaction at a degree comparable to that of DON (Table 2).

In conclusion, enzymatic ribosylamine-5-phosphate formation from ribose-5-phosphate and ammonia is catalyzed by a

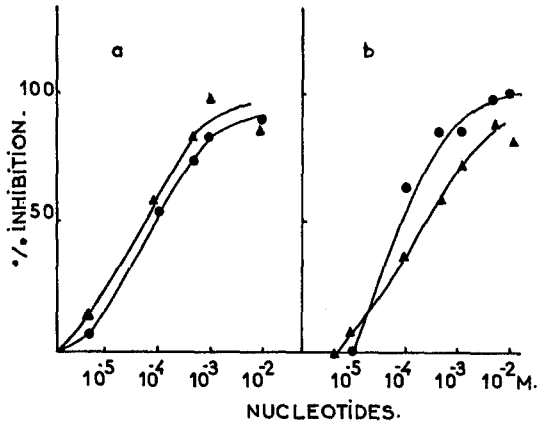

Fig. 3. Feed-back inhibition of PRA synthesis by increasing concentrations of AMP (●) and GMP (▲).
a) PRA synthesis directly from ribose-5-phosphate and ammonia.
b) PRA synthesis from PRPP and glutamine.

native protein present in crude extracts of Escherichia coli B. The glutamine synthetase inhibitor, methionine sulfoximine, has no effect on this reaction which indicates that glutamine is not an intermediate. The relative nitrogen donor activities of ammonia and glutamine in the presence of ribose-5-phosphate and PRPP respectively suggest that the PRA synthesis from ammonia does not proceed with PRPP as an intermediate. The potent feed-back control exerted by purine nucleotides is a further confirmation of the enzymatic nature of the direct ribosylamine-5-phosphate synthesis from ribose-5-phosphate and ammonia.

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