ON THE DISTRIBUTION AND TRANSFORMATION OF THIAMINE AND ITS PHOSPHORIC ESTERS IN BIOLOGICAL MATERIAL

A.Rossi-Fanelli, P.L.Ipata and P.Fasella

Institute of Biological Chemistry University Roma

Received November 22, 1960

In a recent paper (Rossi-Fanelli et al., 1960) we described a method for the quantitative determination of thiamine and its phosphoric esters in biological material. Its application to rat liver gave the following results in ug per mg fresh tissue ± standard error: T[1] = 0.18±0.01; MPT=0.96+0.12; DPT=6.30+0.72; TPT=0.76+0.09. Recoveries were around 90 per cent.

Similar results were successively reported by Rindi and De Giuseppe (1960), who extended the investigation to other rat organs. No quantitative data are available on the distribution of thiamine esters in micro-organisms. It is the object of the present paper to report the results obtained by applying our method to "cell-free" extracts of Mycobacteria. This investigation seemed interesting both to compare the distribution of thiamine phosphoric esters in animal and bacterial cells and as a basis for further metabolic studies on bacteria.

Cells grown on a Sauton culture medium for 1 to 14 days, were harvested by centrifugation and washed three times in cold saline. The cells were disrupted by a refrigerated ball-mill and centrifuged at 18.000xg in the cold. This supernatant (cell-free extract) was treated with 3 volumes of 0.1 N $HClO_4$, neutralized with $KHCO_3$ and centrifuged. The precipitate was discarded and the supernatant divided into two portions. The first was applied on a 3x0.5 cm

---

[1] Abbreviations: T= thiamine; MPT = monophosphothiamine; DPT= diphosphothiamine; TPT = triphosphothiamine; ATP = adenosine triphosphate.

Dowex-50 Column (H^+ form, 300 mesh); TPT was selectively washed out of the column with water, and determined by a standard thiochrome method or by an original manometric method based on its cocarboxylase activity after enzymic conversion into DPT (Rossi-Fanelli et al., 1960). The other portion of the extract was applied on a column made of two superimposed segments (10x1 cm each) of Amberlite IRC-50, 120 mesh; the higher segment was in the Na^+, the lower in the H^+ form. DPT was washed out of the column with water; the two segments were then disconnected; MPT was eluted from the lower and T from the higher segment in 0.1 N HCl. Each isolated compound was determined separately by the thiochrome method. Typical results are reported in Table I.

TABLE I

Quantitative determination of thiamine and its phosphoric esters in cell-free extracts of mycobacteria.

Mycobacteria [1]	Time of growth (days)	T [2]	MPT [2]	DPT [2]	TPT [2]
Lacticola, strain Milch	9 [3]	0.1	1.45	16.0	0.9
"	13 [4]	0.1	0.6	7.0	0.5
Fortuitum	7 [3]	0.1	2.5	11.6	1.3

[1] From the microbiological collection of the "Instituto C. Forlanini", Roma.
[2] Mean values of two determinations expressed as ug/mg of protein N.
[3] Time corresponding to the central part of the logarithmic phase of growth.
[4] Time corresponding to the end of the logarithmic phase of growth.

A comparison with data obtained for rat liver suggests that the four thiamine compounds are present in similar proportions in widely different types of cells. In bacteria, their absolute concentration is much higher (comparable with the value for total thiamine in some yeasts (Cook, 1958) and seems to be dependent on the stage of growth.

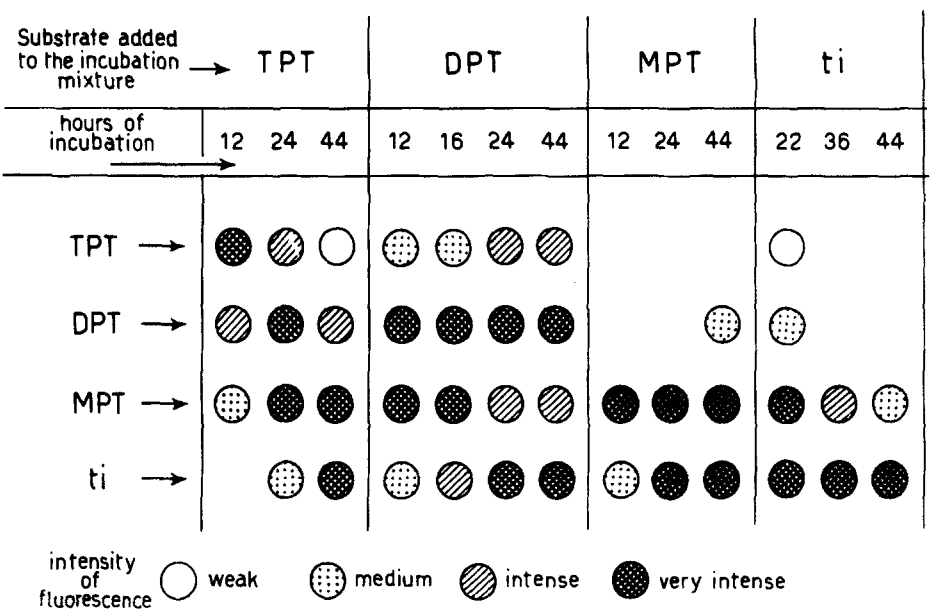
A problem strictly related to the relative concentration of the various thiamine esters is that of their biological transformations. Some information about the latter problem were obtained by the following experiments.

Cell-free extracts were treated with solid ammonium sulfate (500 mg/ml). The precipitate was dissolved in pH 8 tris buffer (0,05 M) and dialized to eliminate the sulfate. The whole procedure was done in the cold. 0.2 ml of the dialized extract, containing about 1% of protein, was incubated at 38°C in a mixture made a: 20 mM T (or T ester), 0.1 ml; pH 8 Tris buffer 0.05 M: 0.1 ml; 0.015 M ATP; 0.1 ml; 0.03 M $MgCl_2$: 0.1 ml; 0.03 M K_2HPO_4: 0.1 ml; water to a final volume of 0.8 ml.

Samples taken at different times were added with two volumes of ethanol and centrifuged; 0.3 ml of the supernatant was analyzed for thiamine and its derivatives according to Siliprandi (1954). The results are shown in fig. 1.

FIGURE I

Action of extracts of Mycobacterium Lacticola, strain Milch, on thiamine and its phosphoric esters.

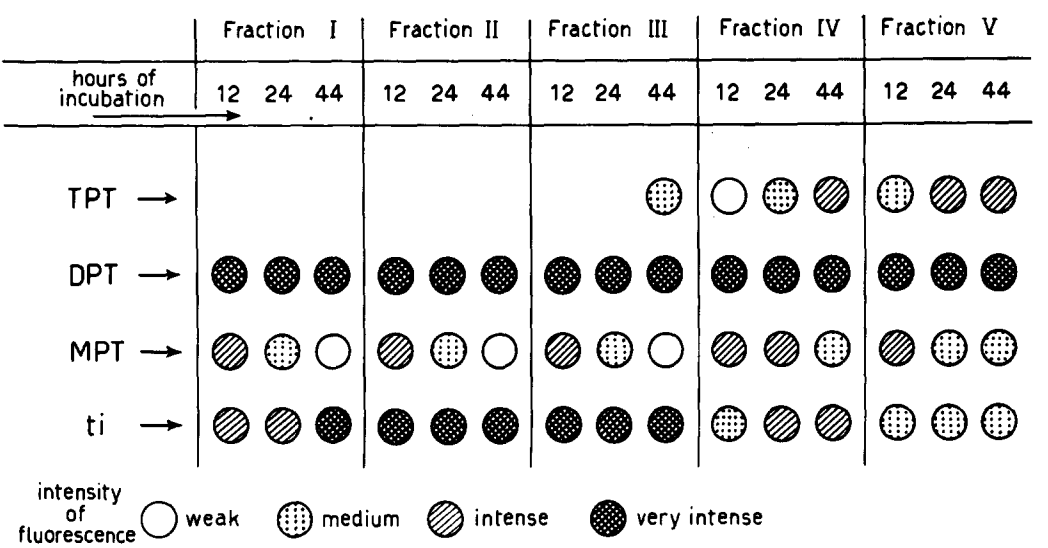


Schematic representation of chromatograms of the incubation mixtures (See text for details).

All T esters are dephosphorylated to inferior esters and T. The most effective substrate for DPT synthesis is obviously T. With MPT as substrate, DPT is formed only after long periods of incubation, i.e. when much of the MPT has been transformed into T, so that it seems probable that DPT is formed from the latter and not from MPT. This is in agreement with current knowledge (Metzler, 1960) on the biosynthesis of DPT in other biological material. It remains to be established if MPT is formed only by dephosphorylation of DPT and TPT or also by direct phosphorylation of T. When discussing the biological significance of the relevant amount of MPT found by us in bacteria, it should also be borne in mind that, according to Camiener et al., (1960), MPT is an intermediate in the biosynthesis of T from its thiazole and pyrimidine moieties. DPT seems to be the only effective precursor of TPT. A similar observation was made by Greiling (1958) with yeast extracts and by Kiessling (1959) with living yeast cells.

FIGURE 2

Action on DPT of the different fractions of cell-free extracts of Mycobacterium Lacticola, strain Milch.



Schematic representation of chromatograms of incubation mixtures (See text for details).

In order to obtain a preliminary purification of the enzyme catalyzing the synthesis of TPT, the extract was fractioned with ammonium sulfate. Each of the fractions was tested for its activity on DPT in the conditions previously described (see figure 2). Fraction I, II and III (precipitated at a final concentration of respectively 100, 200 and 300 mg/ml) contained most of the phosphatasic and little or no phosphorylating activity. Fraction IV and V (obtained at 400 and 500 mg/ml) had much less phosphatasic and marked phosphorylating activity.

Experiments meant to investigate the effect of the compositon of the reaction mixture on the phosphorylating activity of the latter two fractions showed that ATP is necessary for the formation of TPT from DPT; this excludes that TPT may be formed by the reaction: 2 DPT = MPT + TPT. Moreover, it was shown that magnesium ions are not required for TPT synthesis.

REFERENCES

Camiener, G.W., and Brown,G.M., J.Biol.Chem., 235 2404 (1960).
Cook,A.H., The Chemistry and Biology of Yeasts, p. 562 Academic Press, New York (1958).
Greiling,G., Reported at the 4th Intern. Congress of Biochem., Vienna (1958).
Kiessling,K.H., Acta Chem. Scand., Acta Chem. Scand., 13, 1358 (1959)
Metzler, D.E., The Enzymes, 2, 337, Academic Press, New York (1960).
Rindi,G., and De Giuseppe, L., Experientia, 16, 447 (1960).
Rossi-Fanelli,A., Olivo,F., Fasella,P., and Riva.F., Rendiconti Academia dei Lincei, 28, 559 (1960).
Siliprandi, D., and Siliprandi, N., Biochim. et Biophys.Acta, 14, 52 (1954).