AN ENZYMATIC CLEAVAGE AND PHOSPHORYL TRANSFER REACTION INVOLVING MONOTHIOLPHOSPHATE.[1]

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Received February 26, 1962

Thiolphosphates have been suggested as intermediates in various activation processes, but conclusive evidence for their participation in these reactions has not been obtained. Recently, however, cysteamine-S-phosphate has been synthesized and its enzymatic hydrolysis to cysteamine and orthophosphate demonstrated (Akerfeldt, 1959, 1960). Earlier, Binkley (1949) demonstrated the enzymatic cleavage of monothiolphosphate to orthophosphate and sulfide.

While investigating the cleavage of thiolphosphates with enzymes obtained from Escherichia coli, glucose was found to be a potent inhibitor. Evidence presented in this report suggests that an enzymatic phosphoryl transfer from monothiolphosphate to glucose probably accounts for this inhibition.

Materials and Methods

Na_3O_3PS was synthesized from PCl_3 by the method of Yasuda and Lambert (1954) and $Na_3O_3P^{32}S$ by the hydrolysis of $P^{32}SCl_3$ (Radiochemical Center, Amersham, England) by the method of Akerfeldt (1960). Orthophosphate was determined by the method of Fiske and SubbaRow (1925). Thiolphosphate gave no color with the Fiske-SubbaRow reagents and its presence did not interfere with the Fiske-SubbaRow determination of orthophosphate. Thiolphosphate was quantitatively converted to orthophosphate by oxidation with bromine water as

[1]This work was supported by a grant from the Julius and Dorothy Fried Research Foundation.

outlined by Binkley (1949) and then measured as orthophosphate. Phosphoryl transfer from P^{32}-thiolphosphate to glucose was determined after bromine oxidation and separation of the organic and inorganic phosphates by the method of Neilson and Lehninger (1955). Neither glucose-1-P nor glucose-6-P were converted to orthophosphate by the bromine water treatment.

Escherichia coli, Crookes strain, was grown, harvested, and fractionated by methods previously published (Fujimoto and Smith, 1962). The fraction used in these experiments was obtained by precipitation with solid ammonium sulfate between 55 and 90 per cent saturation after previous treatment of the extract with protamine sulfate. In one experiment a fraction obtained by elution from diethylaminoethyl cellulose was used. Glucose-6-phosphate-dehydrogenase was obtained from baker's yeast by the procedure of Horecker and Smyrniotis (1955) and glutathione reductase was generously supplied by Dr. D. E. Atkinson.

Results

The results in Table I demonstrate an enzymatic cleavage of thiolphosphate to orthophosphate. Of several divalent metals tested, manganous and ferrous ions were the most effective in stimulating the reaction. Complete dependence of the cleavage reaction on divalent metals could not be demonstrated. Most of the sulfur released during the reaction could be recovered as sulfide and as elementary sulfur. A variety of compounds, including sodium fluoride, dinitrophenol, potassium arsenate and sodium arsenite, were tested for their inhibitory effect on the reaction. While these compounds were without effect on the reaction glucose and several other hexoses were strongly inhibitory (Table I).

The possibility that the glucose inhibition of the cleavage reaction could be explained by a phosphoryl transfer reaction was tested using P^{32}-labeled thiolphosphate. At the end of the incubation period the reaction was stopped by addition of trichloroacetic acid and the remaining thiolphosphate was converted with bromine water to orthophosphate which was extracted from

TABLE I

Enzymatic Cleavage and Phosphoryl Transfer Involving Monothiolphosphate

System	Pi Produced	P^{32} Transferred	
	μmoles	cpm.	μmoles
1. No additions*	3.65	132	0.03
2. Plus glucose	0.73	3780	0.88
3. No. 2 minus enzyme	0†	264	0.06**

* The complete system contained in a final volume of 1 ml in μmoles: Tris buffer, pH 8.9, 100; P^{32}-labeled thiolphosphate, 10 (4300 cpm/μmole); $MnCl_2$, 1; cysteine, 5; E. coli fraction, 1.15 mg protein. Tube nos. 2 and 3 contained 20 μmoles of glucose.
** A similar control lacking glucose was frequently run with exactly similar results to the above.
† All values were corrected for a constant amount of orthophosphate which contaminated the thiolphosphate preparation.

the reaction mixtures. In the reaction mixture containing glucose, enzyme and P^{32}-thiolphosphate a considerable amount of radioactivity was unextractable while in those mixtures lacking either glucose or enzyme no radioactivity remained (Table I). An additional experiment showed that a large portion (70%) of the unextractable radioactive phosphate compound was converted to orthophosphate by acid hydrolysis (1N HCl at 100°C for 10 minutes).

Further evidence that phosphoryl transfer from thiolphosphate to glucose occurred is shown by the results in Fig. 1. The coupled system used in this experiment included phosphoglucomutase, glucose-6-P dehydrogenase, the E. coli fraction, thiolphosphate, glucose and TPN^+. The increase in absorbance at 340 mμ in the coupled system was dependent upon the addition of thiolphosphate to the reaction mixture. Omission of any single component from the reaction mixture resulted in no observable increase in absorbency at 340 mμ. While several reagents are known to cause spectral shifts with TPN^+, this possibility was eliminated in the coupled reaction since, as shown in Fig. 1, a rapid decrease in absorbency occurred on the addition of oxidized glutathione and a TPNH specific glutathione reductase to the system.

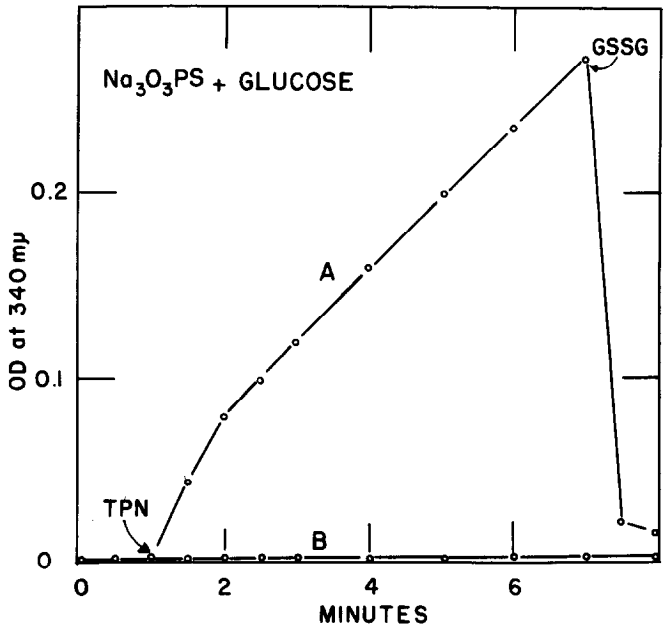

Figure 1. TPNH formation with coupled glucose-6-phosphate dehydrogenase, E. coli system. Complete system contains in μmoles in 3 ml, Tris, pH 8.0, 100; cysteine, 3; $MnCl_2$, 1; glucose, 60; E. coli fraction 1.8 mg; glucose-6-P dehydrogenase, 2 units; and TPN^+, 1 μmole added at 1 minute. Curve A includes thiolphosphate, 5 μmoles. Curve B was obtained when either glucose or thiolphosphate were omitted from the reaction. At 7 minutes 3 units of glutathione reductase and 2 μmoles of oxidized glutathione were added to cuvette A.

Direct evidence for phosphoryl transfer to glucose was obtained using C^{14}-labeled glucose and thiolphosphate with the E. coli enzyme. As shown in Fig. 2, the products of this reaction were chromatographed as their borate complexes on an anion exchange resin (Dowex-I, chloride phase) (Khym and Cohn, 1953). In addition to the unreacted C^{14}-glucose, both C^{14}-glucose-1-P and C^{14}-glucose-6-P in a ratio of 2:1 were found.

Although the thiolphosphate and phosphoramidate transfer systems (Fujimoto and Smith, 1962) are found in similar E. coli fractions, the ratio of these activities varies considerably over purification, and the marked differences in pH optima and fluoride sensitivities of the two systems suggest that they may be different enzymes. Further purification and investigation of the thiolphosphate system is in progress.

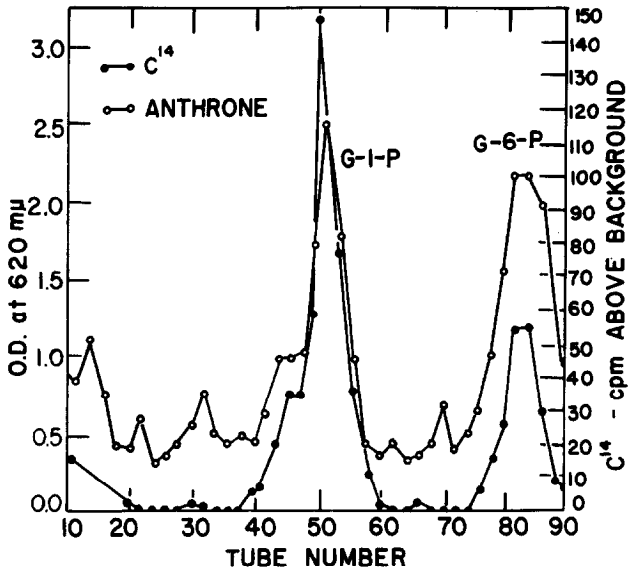

Figure 2. The reaction mixture contained in 2 ml in μmoles: Tris buffer, pH 8.9, 250; thiolphosphate, 25; cysteine, 12.5; $MnCl_2$, 2.5; C^{14}-glucose, 50 (12,200 c.p.m. per μmole) and enzyme, ca. 30 μg. Incubation was at 37°C for 60 minutes. The reaction mixture was poured directly onto a column of Dowex-I-X8 (100-200 mesh 1 x 10 cm in chloride phase) followed by a mixture containing 30 μmoles of glucose-1-P and 20 μmoles of glucose-6-P. The resolution of the phosphate esters was followed by the anthrone method (Morris, 1948) and the samples were also counted for radioactivity.

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