PURE CRYSTALLINE OXYTHIAMINE PHOSPHORIC ESTERS

PREPARATION AND SOME CHEMICAL AND BIOLOGICAL PROPERTIES*

by

F. NAVAZIO, N. SILIPRANDI AND A. ROSSI-FANELLI

Institute of Biochemistry, University of Rome (Italy)

The observation that oxythiamine is a potent thiamine antagonist[1] and that thiamine functions *in vivo* as diphosphate (cocarboxylase[2]) focussed the attention of many workers on the biological effects of the phosphorylated derivatives of oxythiamine[3,4,5,6].

Except for MPOT which, as prepared according to the method of CERECEDO AND EUSEBI[7], yields a pure compound, both DPOT and TPOT when prepared according to the method of the latter authors (ONRUST *et al.*[5], CERECEDO AND EUSEBI[7], VELLUZ *et al.*[8]) prove chromatographically and electrophoretically[9] to be mixtures of many oxythiamine phosphoric esters (MPOT, DPOT, TPOT)**.

In the present paper a description is given of an electrophoretic procedure for obtaining pure crystalline oxythiamine phosphoric esters. Some chemical and enzymic properties of these compounds have also been investigated.

EXPERIMENTAL

Oxythiamine was prepared according to the method of RYDON[10]. Oxythiamine phosphoric esters were prepared from oxythiamine by the procedure described by VISCONTINI *et al.*[11] for the preparation of thiamine phosphoric esters. The resulting mixture (MPOT + DPOT + TPOT) was then resolved into the single components by means of column electrophoresis. The apparatus used for this purpose was the same as described by FLODIN AND PORATH[12] and the procedure was very similar to that previously stated for the separation of thiamine and its phosphoric esters[9] and for the preparation of pure triphosphothiamine[13]. Instead of the sodium acetate buffer, triethylammonium formate buffer pH 5.4, $\mu = 0.05$ has been used, following the suggestion of PORATH[14]. The advantage of this buffer is that it can easily be removed at the end of the experiment, as it is a very volatile substance. 110 mg of a mixture of oxythiamine phosphoric esters were placed on a 60 × 4 cm column packed with cellulose powder. After allowing 120 ml of the buffer to flow so that the mixture reached about half-way down the column, a 50 mA current was applied for 14 hours with the cathode at the bottom and the anode at the top of the column. The column was then disconnected and eluted with the same formate buffer on a fraction collector. The various fractions were analyzed by paper chromatography, using the same solvent described for the separation of thiamine and its phosphoric esters[9]. The darkish spots on the chromatograms were identified by means of a Mineralight short wave lamp. For a more accurate analysis the chromatograms were photographed in U.V. light with the technique described by MARKHAM

* This paper was supported by a grant of the Rockefeller Foundation and C.N.R.

** The following abbreviations are used: OT = Oxythiamine; MPOT = monophosphooxythiamine; DPOT = diphosphooxythiamine; TPOT = triphosphooxythiamine; DPT = diphosphothiamine (cocarboxylase).

References p. 279.

AND SMITH[15]. The separated fractions, each corresponding to one ester, were then evaporated under reduced pressure at a temperature below 45° C. In this way the buffer was almost completely removed. The residue was taken up with 2 ml of water and oxythiamine esters precipitated with a mixture (about 100 ml) of equal parts of acetone and absolute ethyl alcohol. The precipitates were then dissolved again in 2 ml of water and reprecipitated with the same acetone-alcohol. When acidified with 1 or 2 drops of conc. HCl and kept at a low temperature the phosphoric esters of oxythiamine precipitated in crystalline form.

RESULTS

The separation of oxythiamine phosphoric esters by means of the cellulose column electrophoresis is shown in Fig. 1.

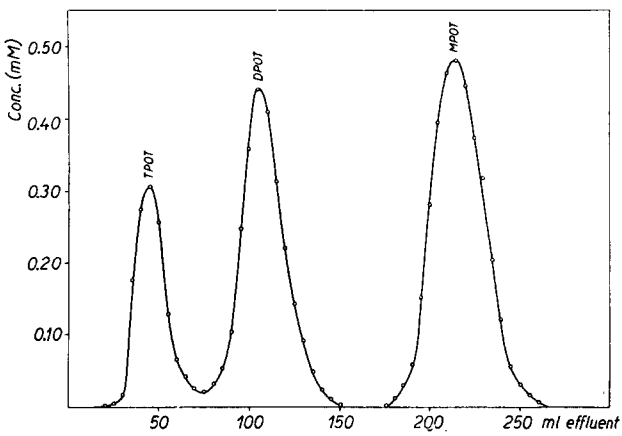


Fig. 1. Separation of oxythiamine phosphoric esters (MPOT + DPOT + TPOT = 120 mg) by cellulose powder column electrophoresis. Triethylammonium formate buffer pH 5.4, 0.05 *M*; 40 mA current for 12 hours.

In Fig. 2 (a, b, c) the photographs of the crystals of respectively MPOT, DPOT and TPOT are reported.

TPOT crystals are hygroscopic, the melting point is at 173°–175° C, and the refractive index (in diethyl-aniline) is 1.5415.

The DPOT crystals are less hygroscopic than those of TPOT, the melting point is at 248°–252° C and the refractive index (in *m*-cresol) is 1.5385.

The MPOT crystals are slightly hygroscopic, the melting point being at 193°–195° and the refractive index (in diethyl-aniline) is 1.5430. The photograph shows their arrangement to be in rose-clustered needles (Fig. 2).

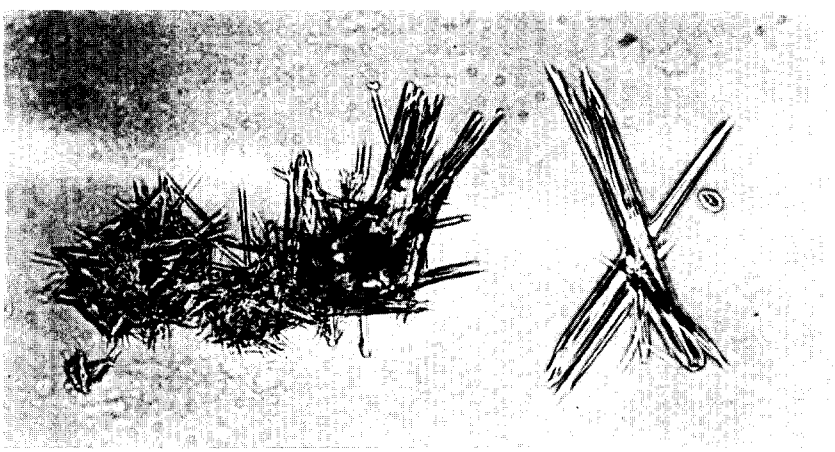
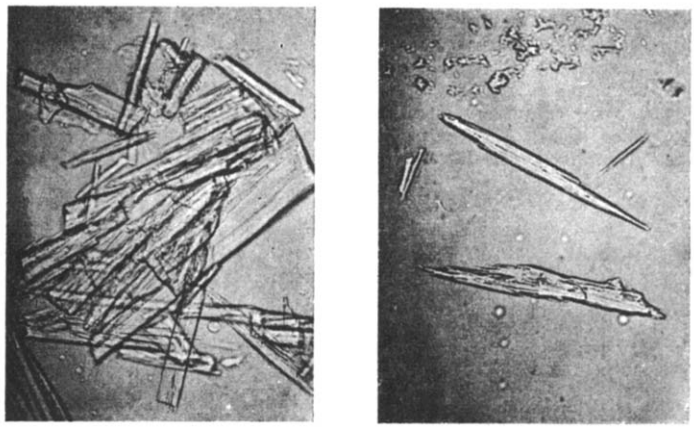


Fig. 2. Crystals of MPOT (a) × 50

References p. 279.



DPOT (b) × 75 and TPOT (c) × 75.

The purity of these esters has been tested chromatographically as also by elementary analysis.

The titration curves of oxythiamine phosphoric esters are shown in Fig. 4.

The U.V. spectra of oxythiamine and of its esters are exactly the same: this means that the phosphoric chain does not affect their absorption in U.V. light (Fig. 5).

An analogous observation has been made for thiamine and its phosphoric esters. The U.V. spectra of oxythiamine and of its esters do not vary with the

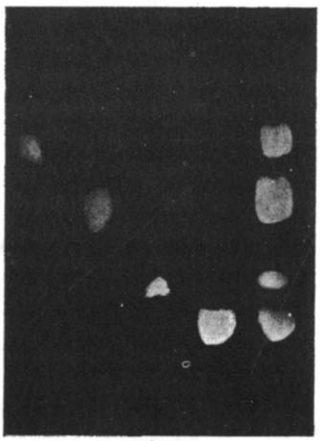


Fig. 3. Chromatogram of oxythiamine phosphoric esters. Solvent: *n*-propanol, water, 1 *M* acetate buffer pH 5 (65:20:15); 30 hours; 150 μg of the mixture and 50 μg of each compound. Munktell paper No. CHR 100[9]. The photograph was taken in U.V. light according to MARKHAM AND SMITH[15].

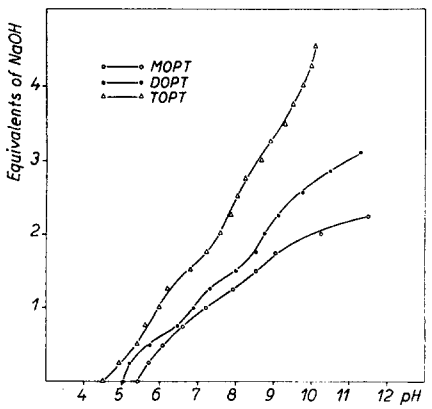


Fig. 4. Titration curves of oxythiamine phosphoric esters.

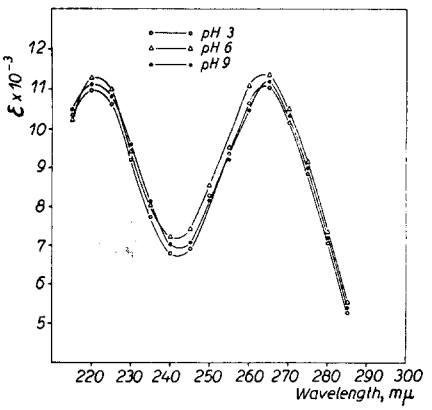


Fig. 5. Absorption spectra of oxythiamine phosphoric esters (MPOT, DPOT and TPOT).

References p. 279.

pH. For thiamine and its phosphoric esters, on the contrary, a considerable variation of the U.V. spectra with the pH has been noted[16].

Kept in dry form in the dessicator at 0° C the oxythiamine esters remained unaltered. The optimum stability in solution was at pH 5. At this pH and at 0° C the esters remained unaltered, except TPOT which began to dissociate into DPOT after 2 or 3 weeks. The phosphoric esters of oxythiamine were much more stable than the esters of thiamine.

THE EFFECTS OF OXYTHIAMINE AND ITS PHOSPHORIC ESTERS ON YEAST CARBOXYLASE

Oxythiamine phosphoric esters have been found to inhibit the enzymes, which bring about the anaerobic and aerobic decarboxylation of pyruvate. EUSEBI AND CERECEDO[3] first demonstrated the inhibition of yeast carboxylase by DPOT. VELLUZ AND HERBAIN[4] obtained the same inhibitory effect with TPOT, while ONRUST *et al.*[5] observed that animal pyruvic oxidase was completely inhibited by the same oxythiamine ester. More recently, EICH AND CERECEDO[6] found that the decarboxylation of pyruvate and the formation of acetylmethylcarbinol by wheat germ carboxylase were inhibited by DPOT and that this inhibition was largely dependent upon the order of addition of the inhibitor and cocarboxylase to the enzyme. Oxythiamine, on the contrary, did not affect pyruvate decarboxylation.

In the present work the effects of oxythiamine and its phosphoric esters, prepared in pure form as described above, have been tested on the yeast carboxylase system.

Yeast carboxylase was prepared according to the method described by GREEN *et al.*[17] as far as and including stage 4. A further purification was achieved by treating the solution of carboxylase with calcium phosphate gel (1 volume of calcium phosphate gel to 5 volume of the enzyme solution). The apocarboxylase was then prepared from the supernatant following the same procedure[17]. Effects of oxythiamine derivatives on carboxylase activity of the reconstructed system (by addition of cocarboxylase) were determined manometrically. The reaction mixture contained 0.2 ml of

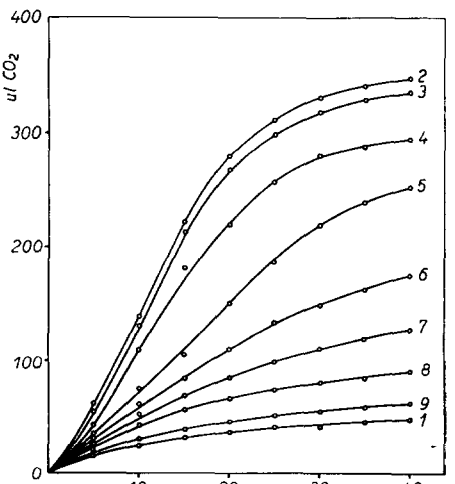


Fig. 6. Effects of OT, MPOT, DPOT and TPOT on yeast carboxylase activity. Curve 1, reaction mixture, no addition. Curve 2, $4 \cdot 10^{-2}$ μM of DPT added with reaction mixture. Curve 3, $4 \cdot 10^{-2}$ μM of DPT in the reaction mixture and $8 \cdot 10^{-2}$ μM of OT or its phosphoric esters added with substrate 20 minutes later. Curve 4, $4 \cdot 10^{-2}$ μM of DPT and $8 \cdot 10^{-2}$ μM of OT and its phosphoric esters with the substrate. Curve 5, $8 \cdot 10^{-2}$ μM of OT with the reaction mixture and $4 \cdot 10^{-2}$ μM of DPT with the substrate 20 minutes later. Curve 6, $8 \cdot 10^{-2}$ μM of MPOT with the reaction mixture and $4 \cdot 10^{-2}$ μM of DPT with the substrate 20 minutes later. Curve 7, $8 \cdot 10^{-2}$ μM of TPOT with the reaction mixture and $4 \cdot 10^{-2}$ μM of DPT with the substrate 20 minutes later. Curve 8, $8 \cdot 10^{-2}$ μM of DPOT with the reaction mixture and $4 \cdot 10^{-2}$ μM of DPT with the substrate 20 minutes later. Curve 9, $8 \cdot 10^{-2}$ μM of DPOT with the reaction mixture and $4 \cdot 10^{-2}$ μM of DPT with the substrate 40 minutes later. Curves 3 and 4 have been calculated from the mean of the values obtained singly with OT and its phosphoric esters. This was possible since the corresponding values are very similar.

References p. 279.

apocarboxylase preparation, 0.3 ml of 0.1% $MnCl_2$, 1 μg of cocarboxylase in 0.1 ml of water, 2.4 ml of phosphate buffer 0.1 M, pH 6.5. 0.2 ml of 0.3 M pyruvate were added to the reaction mixture after temperature equilibration. The reaction temperature was 30° C. When oxythiamine or its phosphoric esters were added, a corresponding volume of the buffer was omitted in the mixture.

The action of cocarboxylase and of oxythiamine derivatives in the carboxylase system was determined, according to EICH AND CERECEDO[6] mainly by the order of addition of the compound to the reaction mixture. The compound added first was placed in the main compartment of the Warburg vessels in contact with the enzyme, the second compound in the side arm was tipped into the main compartment together with the substrate after temperature equilibration.

As EICH AND CERECEDO[6] previously found with the wheat germ carboxylase, when oxythiamine or its phosphoric esters were introduced to the system after cocarboxylase, no inhibition was observed (curve 3). Simultaneous addition of cocarboxylase and oxythiamine or its esters resulted in a slight inhibition (curve 4). The introduction of oxythiamine and of its esters determined an increasing inhibitory action in the following order: OT < MPOT < TPOT < DPOT.

The curve 9 shows that the longer the preincubation time of the inhibitor with the apoenzyme, the more substantial is the inhibition.

These results are generally in agreement with those obtained previously by EICH AND CERECEDO[6] using DPOT. Furthermore they demonstrate that the inhibition by OT derivatives on carboxylase yeast system is dependent upon the number of the esterifying phosphoric radicals. The maximum inhibitory action was developed by DPOT. Since this compound is the analogue of the cocarboxylase (DPT), its inhibitory effect may be interpreted as an antagonism towards the apoenzyme. The lower inhibition determineb dy OT, MPOT and TPOT may therefore be assumed to be dependent upon the necessity of a phosphorylation (OT and MPOT) and of dephosphorylation (TPOT) to DPOT during the incubation in yeast apocarboxylase preparation. It means that OT, MPOT and TPOT probably act on the carboxylase system only if previously transformed into DPOT.

SUMMARY

A procedure for the preparation of the pure crystalline phosphoric esters of oxythiamine (monophospho-oxythiamine, diphospho-oxythiamine and triphospho-oxythiamine) is described. The procedure is based on the electrophoretic separation of a mixture of oxythiamine phosphoric esters, obtained by chemical phosphorylation of oxythiamine.

Some properties of these compounds (U.V. spectra, titration curves, stability in dry form and in solution, chromatographic behaviour on paper) are studied.

The phosphorylated oxythiamine derivatives have been found to inhibit the purified yeast carboxylase system only if added to the apoenzyme before cocarboxylase.

The inhibitory action on carboxylase differs in the various oxythiamine derivatives and increases in the following order: oxythiamine < monophosphooxythiamine < triphosphooxythiamine < diphosphooxythiamine.

RÉSUMÉ

Une méthode de préparation des esters phosphoriques purs cristallisés d'oxythiamine (monophospho-oxythiamine, diphospho-oxythiamine et triphospho-oxythiamine) est décrite. La méthode est fondée sur la séparation par électrophorèse du mélange des esters phosphoriques d'oxythiamine, obtenu par phosphorylation chimique de l'oxythiamine.

References p. 279.

Quelques unes des propriétés de ces corps (spectres U.V., courbes de titrage, stabilité à l'état sec et en solution, comportement chromatographique sur papier) sont étudiées.

Les dérivés phosphorylés de l'oxythiamine ne se sont montrés capables d'inhiber la carboxylase purifiée de la levure que s'ils sont ajoutés à l'apoenzyme avant la cocarboxylase.

Le pouvoir inhibiteur sur la carboxylase diffère pour chaque dérivé de l'oxythiamine et augmente dans l'ordre suivant: oxythiamine < monophosphooxythiamine < triphosphooxythiamine < diphosphooxythiamine.

ZUSAMMENFASSUNG

Es wird eine Herstellungsweise von reinen kristallinischen Oxythiamin-Phosphorestern (Monophospho-oxythiamin, Diphospho-oxythiamin und Triphospho-oxythiamin) beschrieben. Die Methode beruht auf der elektrophoretischen Trennung einer Mischung von Oxythiamin-Phosphorestern, welche durch chemische Phosphorylierung von Oxythiamin erhalten wurden.

Einige Eigenschaften dieser Substanzen werden untersucht: U.V.-Spektrum, Titrierungskurven, Stabilität in trockenem und gelöstem Zustande, Verhalten bei papierchromatographischer Analyse.

Es wurde festgestellt, dass das gereinigte Hefe-Karboxylasesystem nur dann durch die phosphorylierten Oxythiaminderivate gehemmt wird, wenn man letztere vor der Kokarboxylase dem Apoenzym hinzufügt.

Die auf Karboxylase ausgeübte Hemmungswirkung ist je nach dem untersuchten Oxythiaminderivat verschieden; sie steigt in folgender Reihenfolge an: Oxythiamin < Monophosphooxythiamin < Triphosphooxythiamin < Diphosphooxythiamin.

REFERENCES

1 M. SOODAK AND L. R. CERECEDO, *J. Am. Chem. Soc.*, 66 (1944) 1988.
2 R. J. WILLIAMS, R. E. EAKIN, E. BEERSTECHER JR. AND W. SHIVE, *The Biochemistry of B Vitamins*, Reinhold Publ. Corp., 1950, p. 153.
3 A. J. EUSEBI AND L. R. CERECEDO, *Federation Proc.*, 9 (1950) 169.
4 L. VELLUZ AND M. HERBAIN, *J. Biol. Chem.*, 190 (1951) 241.
5 H. ONRUST, A. C. VAN DER LINDEN AND B. C. P. JANSEN, *Enzymologia*, 15 (1953) 351.
6 S. EICH AND L. R. CERECEDO, *J. Biol. Chem.*, 207 (1954) 295.
7 L. R. CERECEDO AND A. J. EUSEBI, *J. Am. Chem. Soc.*, 72 (1950) 5749.
8 L. VELLUZ, G. AMIARD AND J. BARTOS, *Bull. soc. chim. France*, 15 (1948) 871.
9 D. SILIPRANDI AND N. SILIPRANDI, *Biochim. Biophys. Acta*, 14 (1954) 52.
10 H. N. RYDON, *Biochem. J.*, 48 (1951) 383.
11 M. VISCONTINI, G. BONETTI, C. EBNOTER AND P. KARRER, *Helv. Chim. Acta*, 34 (1951) 1384.
12 P. FLODIN AND J. PORATH, *Biochim. Biophys. Acta*, 13 (1954) 175.
13 A. ROSSI-FANELLI, N. SILIPRANDI, D. SILIPRANDI AND P. CICCARONE, *Arch. Biochem. and Biophys.*, 58 (1955) 237.
14 J. PORATH, *Nature*, 175 (1955) 478.
15 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.
16 A. ROSSI-FANELLI, A. SEGRE, D. SILIPRANDI AND N. SILIPRANDI, *Il Farmaco*, 10 (1955) 173.
17 D. E. GREEN, D. HERBERT AND V. SUBRAHMANYAN, *J. Biol. Chem.*, 138 (1941) 327.

Received July 15th, 1955