SEPARATION AND QUANTITATIVE DETERMINATION OF THIAMINE AND THIAMINE PHOSPHORIC ESTERS AND THEIR PREPARATION IN PURE FORM

by

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None of the present methods for the determination of thiamine and its phosphoric esters allows to determine separately thiamine and each of its phosphoric esters (MPT, DPT and TPT***).

With biological assays[1] thiamine and thiamine phosphoric esters are determined altogether.

Chemical methods are based either on the difference of the solubility of the thiochrome resulting from the oxidation of thiamine and its phosphoric esters[2], or on the determination of thiochrome fluorescence before and after treatment with phosphatase, which converts phosphate esters into free thiamine[3]. These methods can be employed for the determination of both the vitamin and its phosphorylated derivatives, but they cannot distinguish between the single esters.

The determination of thiamine phosphoric esters, which are enzymically active, is usually carried out by manometric procedure in which the rate of pyruvic acid decarboxylation is followed. This method is very sensitive and exact[4]. Free thiamine and its phosphoric esters that are not enzymically active (MPT), however, cannot be determined.

Paper chromatography of thiamine and its phosphates developed by SPADONI AND TECCE[5] and by VISCONTINI *et al.*[6] and adapted by ROSSI FANELLI *et al.*[6a] to the detection of thiamine esters in the animal tissues, cannot separate quantitatively DPT from TPT. KIESSLING AND LINDAHL[7] very recently proposed a chromatographic separation in very strong acid solvent. However, using this procedure, we did not succeed in obtaining a completely satisfactory separation.

In this paper we shall describe some procedures for separating and quantitatively determining thiamine and its esters from their mixture.

Chromatographic separation:

1. on filter paper
2. on starch column
3. on ion exchange resin.

Electrophoretic separation:

1. by filter paper
2. *a.* by starch column
   b. by cellulose powder column.

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*** T = thiamine; MPT = monophosphothiamine, DPT = diphosphothiamine; TPT = triphosphothiamine.

References p. 61.

These procedures can be conveniently applied in studying these compounds in biological materials and in isolating pure esters from their synthetically prepared mixtures.

EXPERIMENTAL

The mixture of thiamine phosphoric esters used in all the following experiments was prepared according to VISCONTINI *et al.*[6]

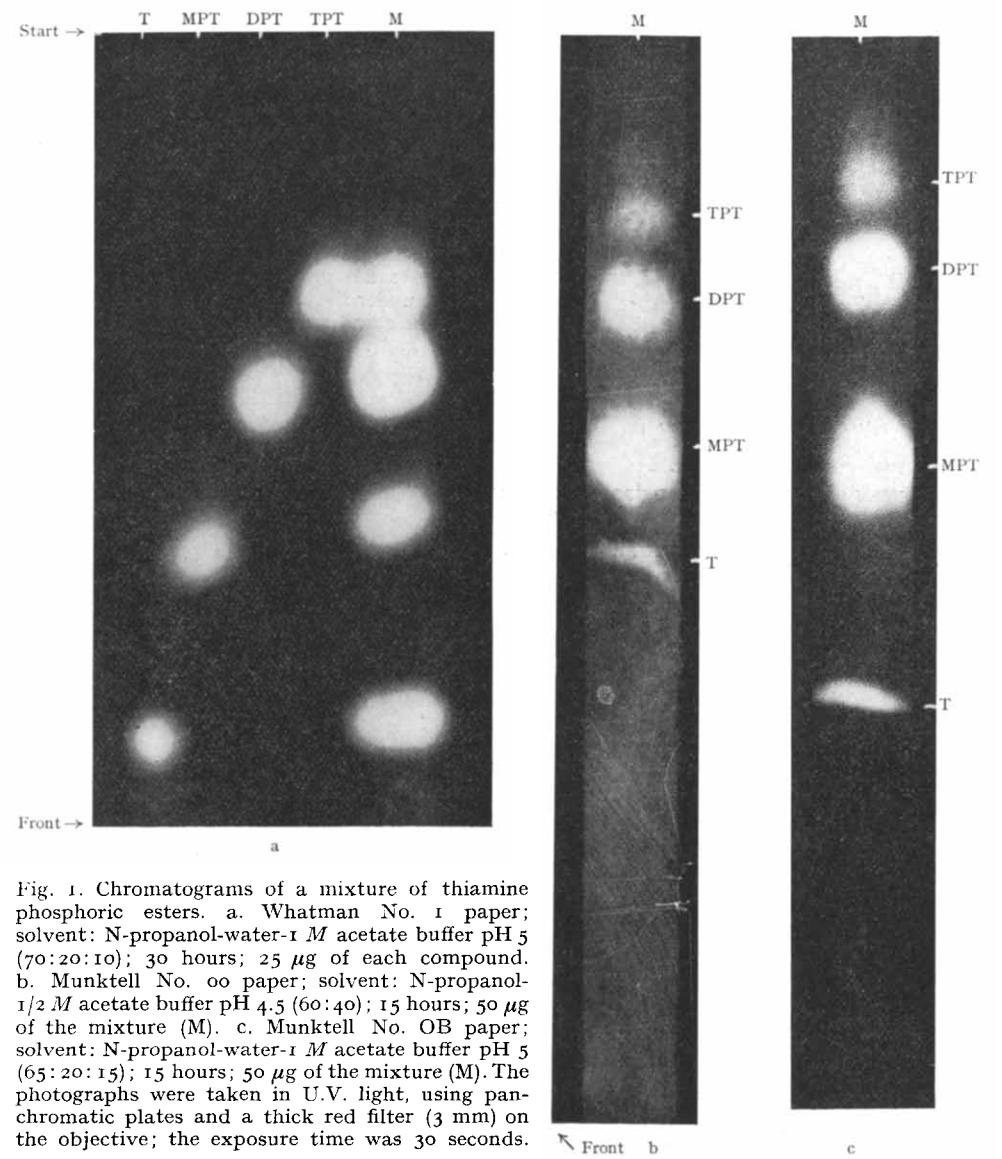

Fig. 1. Chromatograms of a mixture of thiamine phosphoric esters. a. Whatman No. 1 paper; solvent: N-propanol-water-1 *M* acetate buffer pH 5 (70:20:10); 30 hours; 25 μg of each compound. b. Munktell No. 00 paper; solvent: N-propanol-1/2 *M* acetate buffer pH 4.5 (60:40); 15 hours; 50 μg of the mixture (M). c. Munktell No. OB paper; solvent: N-propanol-water-1 *M* acetate buffer pH 5 (65:20:15); 15 hours; 50 μg of the mixture (M). The photographs were taken in U.V. light, using panchromatic plates and a thick red filter (3 mm) on the objective; the exposure time was 30 seconds.

A. *Chromatographic separation*

1. *Paper chromatography.* A good separation of thiamine and its phosphoric esters was obtained using different papers and different solvents as indicated in Fig. 1.

References p. 61.

Munktell papers were used unwashed; with the Whatman No. 1 papers it was necessary to wash successively with 4 *N* HCl, a saturated solution of 8-hydroxyquinoline in 50% ethanol and finally with 50% ethanol[8]. All the papers were run by ascending chromatography over a 15-hour period for Munktell papers and 30 hours for Whatman papers. After drying in a fume chamber at room temperature the papers were sprayed with a mixture of 2 parts of 96% ethanol, 1 part of 10% NaOH and 0.05 parts of 2.5% $K_3Fe(CN)_6$. Thiamine and its esters are in this way transformed into corresponding thiochromes and will appear as fluorescent greenish-blue spots in U.V. light.

No breaking down of the single esters was observed to take place during the experiments (see Fig. 1a), since it was possible to have controls of pure phosphate esters prepared by the procedures described below.

A *quantitative estimation* of the thiamine compounds separated as above is also possible. The papers used for quantitative estimation (for this purpose we generally use Munktell OB paper, but the other papers indicated in Fig. 1 are also suitable) are washed following the HANES AND ISHERWOOD procedure[8]. A known volume of the solution for estimation is placed in small areas 6 cm from the upper edge with an Agla micrometer syringe (Burroughs Wellcome Ltd). Volumes of 10–20 μl of solution containing less than 30 μg of individual substance were used. The spots are dried with a stream of cold air. After running by ascending chromatography for 15 hours, the spots are detected with a "Mineral-light" lamp, without spraying with the alkaline ferricyanide. These spots will appear somewhat dark and are readily observed. Uniform strips, containing each spot, are cut and eluted with water, as described by BRIMLEY AND BARRET[9], by collecting 4 ml of the elution fluid in graduated tubes. This volume is generally sufficient to elute the substance completely, but the elution may be continued in a second tube as a check. The elution liquids are read in the Beckman spectrophotometer at 270 mμ, using as blank the fluid collected by washing, under the same conditions, an identical strip from the same paper. Calculation may be based on the standard reference curves drawn from data as obtained with solutions of the single pure compounds. The single spots are identified by referring to the spots obtained in a parallel run of a known mixture of these pure compounds. It was possible to apply this quantitative estimation to a mixture containing from 10 to 30 μg of each compound with an error of — 5%.

TABLE I

RECOVERY OF T, MPT, DPT AND TPT FROM A MIXTURE SEPARATED BY PAPER CHROMATOGRAPHY

	Applied (μg)	*Recovered (μg)*	*Recovery %*
T	20	19.0	95.0
	20	19.9	99.5
	20	19.3	96.5
MPT	20	19.1	95.5
	20	18.3	91.5
	20	18.8	94.0
DPT	20	19.5	97.5
	20	19.4	97.0
	20	19.1	95.5
TPT	20	19.0	95.0
	20	18.7	93.5
	20	19.4	97.0

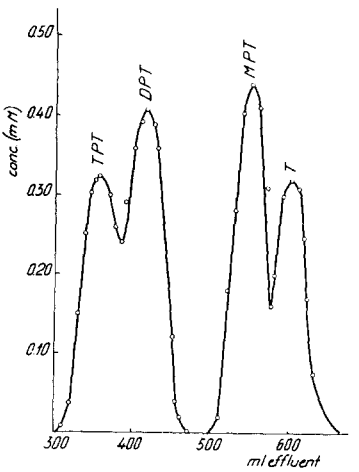

Fig. 2. Chromatographic separation by starch column (100 × 4 cm) of a mixture containing 10 mg of T and 35 mg of a synthetic preparation of MPT, DPT and TPT. Solvent: 0.05 *M* phosphate buffer, pH 5.44.

2. *Chromatography on the starch column.* In order to obtain a resolution of thiamine esters in larger amounts, we attempted to separate them chromatographically on a cellulose powder column. No satisfactory results were obtained in this way. On the other hand, a fairly good resolution was achieved by starch column chromatography.

The starch column used in these experiments was 100 cm in height and 4 cm in diameter and was packed according to FLODIN AND PORATH[10]. The solvent was 0.05 *M* phosphate buffer, pH 5.44. A solution containing 45 mg of the mixture (T, MPT, DPT and TPT) in 4 ml of the buffer was applied on the top of the column. The effluent was collected in a regular series of 5 ml fractions with a flow rate of about 25 ml/hour. The extinction coefficients of the fractions were measured in the Beckman spectrophotometer at 270 mμ. When the concentration of the compounds, expressed in millimoles (*mM*), for each fraction is plotted against the effluent volume, 4 peaks are obtained (Fig. 2).

References p. 61.

The first two peaks, corresponding to TPT and DPT, are completely separated from the last two peaks containing MPT and T respectively. The compounds in each pair of peaks, however, are not quantitatively separated.

The identification of the compounds was carried out with the help of the paper chromatography as described previously. It was necessary, therefore, to desalt the solution beforehand from the inorganic phosphates of the buffer, in order to avoid hydrolysis of the esters during evaporation of the fractions. This evaporation was performed to obtain a suitable concentration of the solution for the paper chromatographic analysis. The presence of the inorganic salts, moreover, markedly alters the position of the spots on the paper. When desalted, the fractions can be conveniently evaporated under reduced pressure at 30°–40° C until the favourable concentration for chromatographing on the paper is obtained.

The same starch cannot generally be used for more than one experiment; a longer contact with the phosphate buffer alters the structure of the starch granules and the column will become wrinkled and discontinuous in some places. In order to prevent moulds from growing in the solvent, a few drops of toluene, or other suitable substances, may be added.

Desalting procedure. The fractions recovered from the starch column were percolated through small columns containing carbon, "Carbo Activ" pretreated with cholesteryl stereate (100 mg per g carbon), in order to adsorb the thiamine compounds. Cellulose powder was mixed in the column with the pretreated carbon to obtain a faster flow rate. The amount of the carbon in the column must be in excess with respect to the amount of thiamine derivatives to be adsorbed. From the specific retention volume (determined with the frontal analysis, see Fig. 3), for a mixture of T, MPT, DPT and TPT, it was found that 1 g of pretreated carbon adsorbed 39 mg of the mixture.

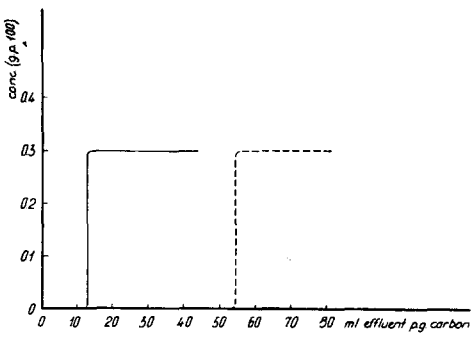

Fig. 3. Frontal analysis diagrams of a 0.3% mixture of T, MPT, DPT and TPT on "Carbo Activ" ---- and on "Carbo Activ" pretreated with cholesteryl stearate (100 mg/g) (———)

After washing with distilled water, which removes the inorganic salts still retained on the column, the thiamine compounds were eluted with 50% ethanol.

The treatment of the carbon with cholesteryl stearate was necessary in order to make the adsorption reversible. We have noticed that when thiamine esters are adsorbed on pure carbon from water solutions it is possible to elute them completely with 50% ethanol. When, however, these compounds are adsorbed from saline solutions, as in the present case, T, MPT, and DPT but not TPT, can be eluted. This fact agrees with the earlier observation by HAGDAHL AND TISELIUS that inorganic salts increase about fourfold the adsorption capacity of the carbon towards some amino acids[11]. Moreover HAGDAHL *et al.*[12] proposed to decrease the adsorption capacity of the carbon by treating it with strongly adsorbable compounds such as hexanol or cholesteryl stearate, thus eliminating the irreversible adsorption on the most active sites. Following this principle we tested carbon samples treated with different amounts of hexanol and cholesteryl stearate and found that the optimum adsorption was represented by carbon treated with cholesteryl stearate. The different adsorption affinities of the thiamine compounds on pure and on pretreated carbon were measured by means of the frontal analysis in the Claesson-Tiselius micro-interferometer (see Fig. 3).

The pretreatment of the carbon was effected as follows: one hundred mg of cholesteryl stearate, dissolved in a 1:1 ethanol-ether mixture, was adsorbed on one g of carbon by shaking the mixture for 48 hours. The ethanol-ether mixture was then diluted with water so that all the cholesteryl stearate was taken up by the carbon.

3. *Chromatographic separation by ion exchanger.* In preliminary assays we found that T and MPT are selectively and quantitatively taken up from a mixture of T, MPT, DPT and TPT by carboxylic acid exchanger (Amberlite IRC 50) in the hydrogen form. T and MPT can be quantitatively removed from the resin on regenerating it with 0.1 *N* HCl. In column experiments we applied the thiamine phosphate ester mixture at the top of the column and washed with water. The TPT was not affected by the resin, but passed down with the dead volume, whereas the DPT was "retarded' by the column and appeared in the effluent after the TPT as a single well-separated peak. Thus, by the above procedure TPT and DPT could be completely separated.

In the experiment reported here we succeeded in separating all the components of the mixture by applying to this chromatographic procedure the *gradient elution technique.* The latter consists of the use of a continuously changing eluant medium, which is produced external to the column in a mixing chamber[13].

HAGDAHL'S column with the coupled filters and mixer[14] was used instead of the usual glass

References p. 61.

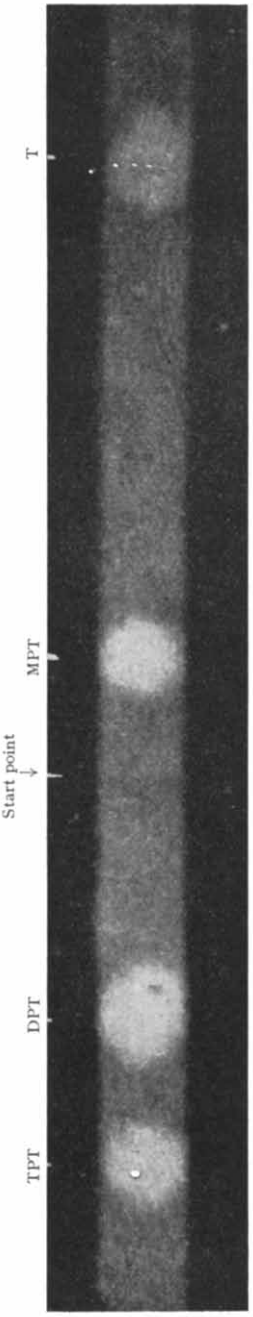

Fig. 5. Separation of a mixture of thiamine phosphoric esters by paper electrophoresis. 50 μg of each compound. Acetate buffer at pH 5.44, μ 0.05. 7.30 hours at a current of 3 mA. Munktell 20 paper.

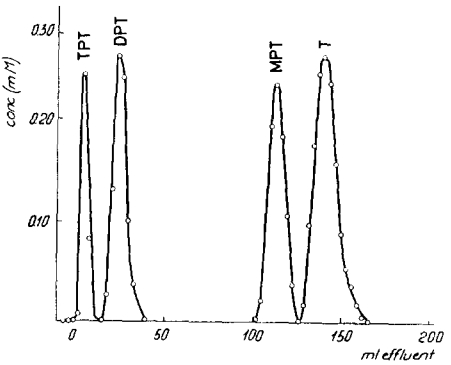

Fig. 4. Ion-exchange separation of a mixture containing 4 mg of T and 8 mg of a synthetic preparation of MPT, DPT and TPT. Exchanger: Amberlite IRC 50, 200–400 mesh, 24 cm^2 in Hagdahl's coupled filters column (1.3 × 18.6 cm), hydrogenform. Flow rate 10 ml/hour. Gradient elution with water–0.05 *N* HCl.

column to improve the chromatographic separation. In a typical experiment three HAGDAHL's filters of 8 ml capacity and lined with polythene were packed with Amberlite IRC 50, mesh 200–400, treated beforehand with 2 *N* NaOH and successively thereafter with 1 *N* HCl and water. After coupling the filters in the column and washing them again with distilled water, 12 mg of the thiamine mixture in 1 ml of water were applied at the top of the column: the mixing chamber, initially containing water, was placed under the one with 0.05 *N* HCl. A slight pressure was applied at the beginning of the experiment in order to quicken the flow rate, which was regulated at 10 ml/hour. Two and one half ml fractions were collected and the extinction coefficients determined at 270 mμ. All 4 components, as indicated in Fig. 4, were separated and recovered quantitatively.

If the usual glass column is used instead of HAGDAHL's coupled filters and if ordinary elution with 0.01 *N* HCl is adopted instead of gradient elution, MPT and T cannot be completely separated.

B. *Electrophoretic separation*

1. *Paper electrophoresis.* The separation of thiamine and its phosphoric esters has been successfully effected also by paper electrophoresis.

The best conditions were obtained with acetate buffer (pH 5.44, $\mu = 0.05$), a relatively hard thick paper, such as Munktell 20, and 3.5 mA current for 6–7 hours. The mixture to be separated was applied on a paper sheet in one spot (0.5 cm) about half-way between the electrodes, the paper being first dipped in the acetate buffer and pressed slightly between two filter papers to remove most of the liquid.

For qualitative purposes it was convenient to place the paper sheet between two glass plates held together by metal clamps. The electrophoresis was carried out at the room temperature. After the current had been applied for the desired length of time, the paper was removed from the glass plates, dried at the room temperature in a fume chamber and sprayed with the alkaline ferricyanide as described for the paper chromatographic method. The spots were detected with the "Mineral-light". Thiamine and MPT move towards the cathode, TPT and DPT towards the anode (see Fig. 5).

The same results have been obtained when phosphate or succinate buffer was substituted for the acetate, while maintaining the same pH and ionic strength.

No breakdown of the pure compounds, when run separately, was observed during the electrophoresis. For this reason and because the spots are very well defined without tailing, the electrophoretic separation can be usefully applied for the *quantitative estimation* of these compounds.

For this purpose the electrophoresis is run with the paper hanging in a closed chamber between the electrode vessels, while maintaining all the other preceding conditions. When the amount of the thiamine mixture to be separated and estimated is in the range between 10–100 μg the mixture is

References p. 61.

applied on one spot in 0.01–0.02 ml solution. By distributing the solution uniformly along a narrow strip across the paper sheet, it is possible to separate larger amounts (from 100 to 2,000 μg or more depending on the width of the paper sheet). The Fig. 6 gives an example of such a separation. The spots or the bands may be identified in UV light without spraying them with alkaline ferricyanide. The elution and the subsequent determination of the extinction coefficients are carried out as in paper chromatography. The error can be readily limited to less than — 5% (see Table II).

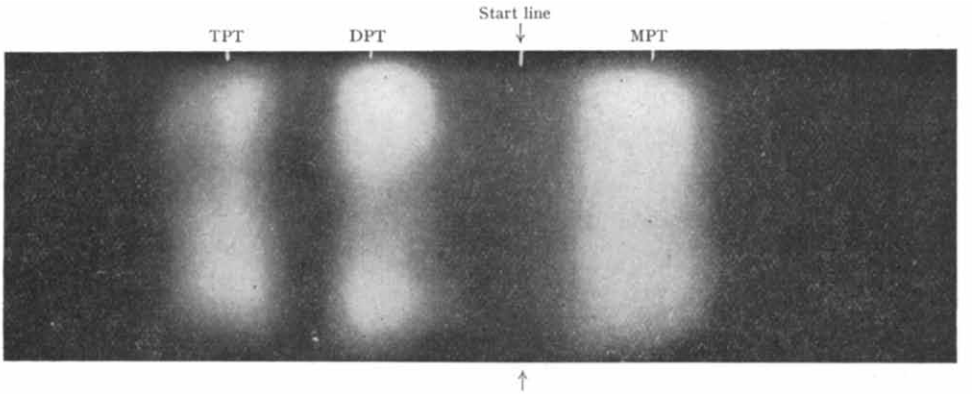

Fig. 6. Separation of a 2 mg synthetically prepared mixture of MPT, DPT and TPT by paper electrophoresis. Acetate buffer at pH 5.44, μ = 0.05. 6 hours at a current of 3 mA. Munktell 20 paper.

TABLE II

RECOVERY OF T, MPT, DPT AND TPT FROM A MIXTURE SEPARATED BY ELECTROPHORESIS ON PAPER

	Applied (μg)	*Recovered (μg)*	*Recovery (%)*
T	50	49.2	98.4
	50	48.8	97.6
	50	47.9	95.8
MPT	50	46.1	92.2
	50	47.7	95.4
	50	47.9	95.8
DPT	50	49.2	98.4
	50	48.3	96.6
	50	49.0	98.0
TPT	50	49.0	98.0
	50	48.2	96.4
	50	49.3	98.6

2. *Column electrophoresis.* The column electrophoresis procedure as described by FLODIN AND PORATH[10] for proteins has also been successfully applied to the separation of thiamine and its phosphoric esters. Both starch and cellulose powder can be used as support for this purpose, even in preparative scale.

a. *Starch column electrophoresis.* The apparatus design and technical details were followed according to FLODIN AND PORATH. As in the paper electrophoresis method, acetate buffer (pH 5.44, μ = 0.05) was used. The mixture of the thiamine derivatives (from 10 to 30 mg) in 2 ml of the buffer was applied at the top of the column (50 × 3 cm). Since TPT and DPT move towards the anode, while MPT and T towards the cathode, the current must not be applied before the substances have reached the approximate center of the column. For this reason the dead volume must be determined beforehand. In our column the dead volume was 115 ml, therefore, after the application of the solution on the column, about 50 ml of the buffer was allowed to flow through before applying

References p. 61.

the current. The top of the column was connected to the cathode, the bottom to the anode and a current of 17 mA for 12 hours was applied. After the current was broken, the column was disconnected from the electrophoresis apparatus and was adapted to the fraction collector. The elution, carried out with the acetate buffer, was regulated to give a flow rate of about 20 ml/hour and the effluent was collected in a series of 3 ml fractions. The estimation of the extinction coefficients and the identification of the fractions was effected as described in the section A, 2.

Fig. 7 reproduces the separation of thiamine derivatives obtained with this procedure from 20 mg of the mixture. Since in the starch column the thiamine esters are adsorbed differently (see Fig. 2), the separation obtained in this way is not entirely due to the electrophoresis itself, but is in part also a result of the adsorption processes on the starch.

As mentioned in the starch chromatography section, mould growth may be avoided by adding a few drops of toluene to the acetate buffer.

b. *Cellulose powder column electrophoresis.* The use of cellulose powder as support in column electrophoresis gave a clearcut and quite quantitative separation of thiamine and its phosphates.

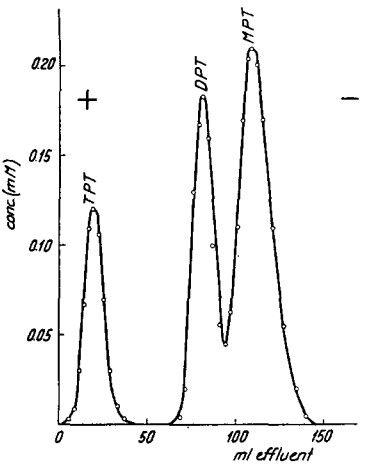

Fig. 7. Separation of a mixture containing 20 mg of a synthetic preparation of MPT, DPT and TPT, by starch column electrophoresis (acetate buffer pH 5.44, $\mu = 0.05$; 17 mA current for 12 hours).

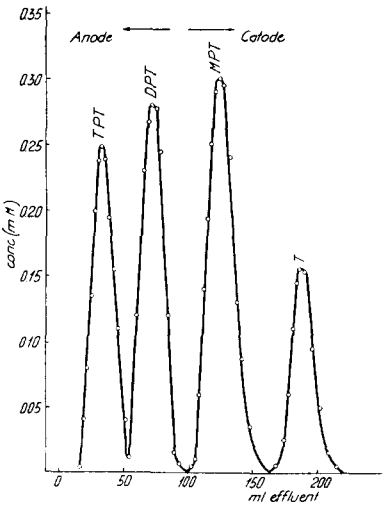

Fig. 8. Separation of a mixture containing 8 mg of T and 27 mg of a synthetic preparation of MPT, DPT and TPT by cellulose powder column electrophoresis (acetate buffer pH 5.44, $\mu = 0.05$; 30 mA current for 15 hours).

The column was prepared as suggested by FLODIN: Whatman ashless powdered cellulose was suspended in acetate buffer (pH 5.44, $\mu = 0.05$). After complete exhaustion of the air by evacuation, the slurry was poured with pressure into a glass electrophoretic column (50 × 3 cm). Thirty-five mg of the thiamine esters mixture, dissolved in 2.5 ml of buffer, were placed on the top of the column. The current (30 mA during 15 hours) was applied after 70 ml of the solvent were allowed to flow down (the dead volume of the column being 150 ml). At the completion of the run the column was disconnected from the electrode vessels and the elution was carried out with the acetate buffer. Fractions of 3 ml each, using a flow rate of 25 ml per hour, were taken by means of a fraction collector and analysed as usual. Quantitative recoveries (100 ± 7% on the average) were obtained for each component from the integration of the elution curves.

Phosphorus analysis and stability of DPT and TPT preparations

The stability of aqueous solutions of DPT and TPT, kept at room temperature at different pH, was tested periodically by paper chromatography. Both the esters were found to be quite stable over 5 days period, in the pH range between 2 and 6. At pH 2.5 DPT and TPT are stable for a longer period.

References p. 61.

TABLE III

P ANALYSIS OF DPT AND TPT. METHOD OF BERENBLUM AND CHAIN[15]

Hydrolysable P was calculated from P values obtained before and after hydrolysis with 1 *N* HCl at 100° for 10 minutes. All these values are referred as per cent of the weight of the compounds.

	Total P	*Inorganic P*	*Hydrolysable P*
TPT calc.	17.8	0.0	11.0
found	18.0	0.2	11.5
DPT calc.	14.0	0.0	7.0
found	14.2	0.1	7.3

Enzymic assay of DPT and TPT

DPT when coupled with the apocarboxylase, prepared from brewers yeast, forms the enzyme carboxylase, which is known to catalyse the reaction:

$$CH_3COCOOH \longrightarrow CH_3COH + CO_2$$

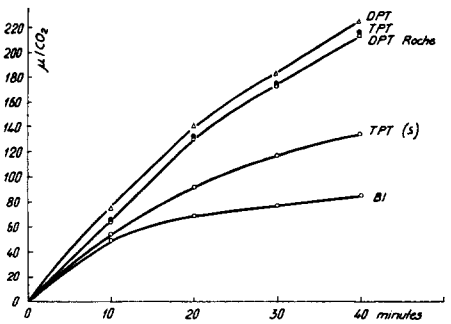

Fig. 9. CO_2 production during a determination of enzymic activity as yeast cocarboxylase of: "H. La Roche" DPT (□), chromatographically pure DPT (△) and TPT (●) and synthetically prepared TPT (○). 0.2 µg of "La Roche" DPT and equivalent amounts of the other preparations were used. Blank = (Bl).

The manometric measurement in a Warburg apparatus of the CO_2 evolved from this reaction gives an exact indication of the carboxylase activity. Hence, by using an excess of apocarboxylase, the cocarboxylase activity is readily determined.

Following this principle we tested the cocarboxylase activity of our DPT and TPT preparations. The procedure described by WESTENBRINK[4] has been employed. As a reference coenzyme, "H. La Roche cocarboxylase" was used. The activity of 0.2 µg of this product and of the equivalent amount* of our DPT and TPT was determined. According to the CO_2 production curves shown in Fig. 9, our DPT and TPT possess respectively an activity of 110 and 103% with respect to the "La Roche" sample.

DISCUSSION

It is shown that chromatographic and electrophoretic procedures have several useful applications in the study of thiamine and its phosphoric esters.

Paper chromatography and paper electrophoresis can be conveniently applied to the detection and determination of thiamine and its phosphoric esters in biological materials. Both methods can be used for routine separation of the esters in 10–100 µg mixture. For larger amounts, up to 2 mg, only paper electrophoresis is suitable.

* The equivalence of our preparations with respect to "La Roche cocarboxylase" was established from the extinction coefficients at 270 mµ.

References p. 61.

Starch chromatography can be conveniently applied when only the separation between the enzymically active compounds (DPT and TPT) and the inactive ones (MPT and T) is required.

Column electrophoresis, with cellulose powder as support, can be employed for the complete separation and determination of thiamine and its esters in a mixture.

Ion-exchange chromatography, with the application of the gradient elution technique, serves the same purpose. This procedure, moreover, is more amenable since the desalting step is avoided.

These procedures led us to prepare pure DPT and TPT, both highly active as yeast cocarboxylase.

In earlier experiments with the TPT prepared synthetically, VELLUZ *et al.*[16] found that TPT has only 80% of the activity of the DPT. The purity of TPT was tested by these authors by the elementary analysis (determination of total C, N, O, H, P, inorganic P and acid-hydrolysable P). This analysis, however, is not unequivocal, being affected by the error due to the presence of inorganic pyrophosphate and MPT accompanying TPT as breakdown products. Pure TPT and equimolecular mixture of MPT and inorganic pyrophosphate give exactly the same results when examined by elementary analysis or by determining the P fractions. We have never succeeded in preparing chromatographically pure TPT by synthesis methods: a mixture of the different thiamine esters was obtained, though a determination of the P fractions showed a good agreement with the theoretically calculated values for pure TPT. Comparison of cocarboxylasic activity between TPT, prepared synthetically, and chromatographically pure TPT, showed that the former has not more than 45% of the activity of the latter. These facts demonstrate that by synthetic methods it is very difficult to obtain quite pure TPT; generally a mixture of TPT, DPT and MPT is obtained.

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SUMMARY

Different methods are described for the separation and determination of thiamine and its phosphoric esters (TPT, DPT and MPT):

1. Chromatographic procedures on paper, and on starch and ion-exchange resin columns.
2. Electrophoretic procedures by paper and by starch and cellulose powder columns.

Some of these procedures were successfully used for preparative purposes.

The enzymic activity, as yeast cocarboxylase, of the isolated DPT and TPT was tested.

RÉSUMÉ

Différentes méthodes sont décrites pour la séparation et le dosage de la thiamine et de ses esters phosphoriques (TPT, DPT, et MPT).

References p. 61.

1. Méthodes chromatographiques sur papier et sur colonnes d'amidon et de résines échangeur d'ions.

2. Méthodes par électrophorèse sur papier et sur colonne d'amidon et de poudre de cellulose.

Certaines des méthodes étudiées ont été utilisées avec succès dans des buts préparatifs. Les activités enzymatiques en tant que cocarboxylase de la levure, du DPT et du TPT isolés ont été vérifiées.

ZUSAMMENFASSUNG

Es wurden verschiedene Methoden zur Abtrennung and Bestimmung von Thiamin and seiner Phosphorsäureester (TPT, DPT und MPT) beschrieben:

1. Chromatographische Verfahren mit Papier, Stärke- und Ionenaustauscherharzsäulen.

2. Elektrophoretische Verfahren mit Papier, Stärke- und Cellulosepulversäulen.

Einige dieser Verfahren wurden erfolgreich zu präparativen Zwecken verwandt.

Die enzymatische Aktivität, wie Hefecocarboxylase der isolierten DPT und TPT wurde geprüft.

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