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THE MANOMETRIC DETERMINATION OF THIAMINE PYROPHOSPHATE AND THE INHIBITION OF THE ACID YEAST PHOSPHATASE*

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SUMMARY

Sodium molybdate is a powerful inhibitor of the acid yeast phosphatase in both fresh baker's yeast and dried brewer's yeast, provided that the yeast is suspended in a suitable buffer. It displays no action in citrate or phosphate buffers, but is active in acetate or maleate buffers, both at the optimum pH of 3.7 of the phosphatase and at pH 6.2. Depending upon the relative concentrations of yeast and phosphate ester substrate, either 0.1 m*M* or 1 m*M* molybdate gives adequate protection against hydrolysis.

In the manometric determination of thiamine pyrophosphate molybdate can replace thiamine as inhibitor of acid phosphatase with full retention of sensitivity if the assay is performed in 0.1 *M* maleate (pH 6.2) instead of 0.1 *M* phosphate (pH 6.2).

In studies of the phosphorylation of thiamine and its antagonists by ATP molybdate has also proved to be useful for inhibiting the acid phosphatase present in preparations of yeast thiaminokinase.

INTRODUCTION

In the manometric determination of TPP this compound is added to an excess of dried brewer's yeast, from which the TPP originally present has been removed by treatment mildly-alkaline reagents, while the carboxylase enzyme protein stays behind in the yeast residue[1]. The rate of production of CO_2 from added pyruvate is then measured; it is proportionate to the amount of TPP present[2,3]. Furthermore, thiamine is also added to the alkaline-washed yeast; it inhibits the acid yeast phosphatase (optimum pH 3.7–4.0) and thus prevents hydrolysis of the added TPP (see ref. 4). This makes the method much more sensitive[5].

The amount of thiamine used is in large excess as compared to TPP: for instance, a Warburg vessel may contain 50 μg of thiamine, together with 0.05 μg TPP. Some

Abbreviations: OTPP, oxythiamine pyrophosphate; TCA, trichloroacetic acid; TPP, thiamine pyrophosphate.

* This work forms part of investigations on the metabolism of thiamine by H. G. K. WESTENBRINK and collaborators.

investigators have raised objections against this procedure; one of them being that additional TPP may be produced from the thiamine by thiaminokinase—also present in the yeast—in reaction mixtures also containing ATP (see ref. 6). In our experience there is little need for this apprehension; under the conditions obtaining in the determination of TPP such an additional synthesis is very small. Moreover, complications of this kind can be avoided by substituting the pyrimidine moiety of the vitamin* for thiamine.

Nevertheless, circumstances may sometimes occur which make the use of thiamine as an inhibitor of the acid yeast phosphatase definitely undesirable or even impossible. Such was the case in this laboratory during a study of the pyrophosphorylation of the thiamine antagonists, pyrithiamine and oxythiamine, by ATP and a preparation of thiaminokinase from yeast[7]. Only with great difficulty can thiaminokinase preparations be obtained free of acid phosphatase[8], so usually an inhibitor of the latter has to be added to the reaction mixture. In the studies mentioned above this could not be thiamine, as it is preferentially phosphorylated. Nor could thiamine be replaced by its pyrimidine moiety, for this is a less potent inhibitor, and the amounts required were so large that they also inhibited the thiaminokinase.

We have therefore searched for another inhibitor of the acid yeast phosphatase, not structurally related to TPP. The work of ROTHSTEIN[9] indicated that molybdate might be suitable, and this has indeed proved to be the case. The present report will relate under which conditions molybdate may be used instead of thiamine in the determination of TPP, and will draw attention to certain peculiarities inherent in its action as an inhibitor of acid yeast phosphatase.

MATERIALS AND METHODS

The yeasts used were a commercially available fresh baker's yeast ("Koningsgist", Delft) and brewer's yeast, air-dried at room temperature, prepared by the "Oranjeboom" Brewery, Rotterdam.

TPP and thiamine were obtained from Hoffman-La Roche (Basle, Switzerland); ATP (chromatographically pure, disodium salt) from Schwartz, Inc. (New York); β-glycerophosphate (less than 0.1% α-isomer) from Eastman Kodak, Inc. (Rochester, N.Y.). The other chemicals used were different commercial brands of analytical-reagent quality.

Acid phosphatase activity was determined by incubating the reaction mixture —containing yeast preparation, substrate, and other additions in a suitable buffer— at 28°, removing samples at certain times and pipetting each into an equal volume of 10%TCA. Aliquots of the filtrates were assayed for inorganic phosphate. When TPP was the substrate, the reaction was usually followed by determining the remaining TPP. In those cases, 1 ml samples of the reaction mixture were mixed with 5 ml boiling 0.04 N HCl, containing 1 mg of thiamine, and boiled for 1 min. After neutralizing to pH 6.2 and centrifuging to remove precipitated protein, aliquots of the filtrate were assayed for TPP.

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* 2-Methyl-4-amino-5-aminomethyl pyrimidine or 2-methyl-4-amino-5-aethoxymethylpyrimidine.

TPP was determined as described by WESTENBRINK AND STEYN-PARVÉ[10]. Inorganic phosphate was determined according to SUMNER[11]. When determining phosphate liberated by hydrolysis of TPP, the latter compound was first removed by adsorbing it on to Bentonite at pH 2–3 (see ref. 12), as it interferes seriously with the determination.

EXPERIMENTS AND RESULTS

Replacement of thiamine by molybdate in the determination of TPP

When sodium molybdate (10^{-4} *M*) was added to alkaline-washed dried brewer's yeast instead of thiamine (100 μg/ml) and standard amounts of TPP were tested in the usual manner[10], very low calibration curves were obtained (see Fig. 1, curves A

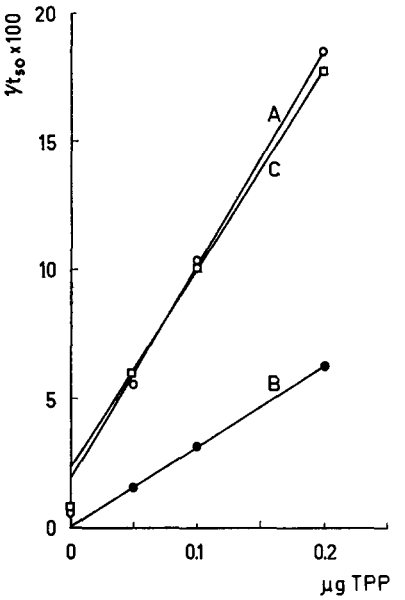

Fig. 1. Calibration curves for the determination of TPP. A, in 0.1 *M* phosphate (pH 6.2) with thiamine (○--○); B, in 0.1 *M* phosphate (pH 6.2) with 10^{-4} *M* molybdate (●—●); C, in 0.1 *M* maleate (pH 6.2) with thiamine (□—□); $1/t_{50}$, reciprocal of time in which 50 μl CO_2 is produced.

and B). Evidently there was very little inhibition of the acid yeast phosphatase under these circumstances. It was clear that thiamine could not simply be replaced by molybdate and a more thorough investigation of the conditions required for the in-inhibitory action of molybdate was indicated.

Inhibition of acid yeast phosphatase by molybdate

Fresh baker's yeast, which has a high content of acid phosphatase, was used as a source of the enzyme in the first series of experiments. β-Glycerophosphate served as a substrate. As can be seen from the first 2 experiments of Table I, there was no indication that molybdate inhibited the hydrolysis of β-glycerophosphate at the

optimum pH of 3.7 in 0.1 *M* citrate buffer, although the ratio of substrate and molybdate was varied.

Now ROTHSTEIN never used any buffer in the experiments in which he demonstrated an inhibition of acid yeast phosphatase by molybdate. Moreover, he incubated the yeast with inhibitor for 15 min before adding substrate, and stated that under these circumstances citrate was also slightly inhibitory[9]. Accordingly, we carried

TABLE I

ACTION OF MOLYBDATE UPON ACID YEAST PHOSPHATASE IN DIFFERENT BUFFERS

Source of acid phosphatase: fresh baker's yeast 10 mg/ml (fresh, pressed weight). Buffers: 0.1 *M* citrate or 0.1 *M* acetate, pH 3.7. Molybdate added as 0.01 *M* sodium molybdate. Incubation at 28°. Reaction stopped by adding an equal volume of 10% TCA. All determinations in duplicate.

Expt. No.	*Substrate*	*Concentration (M)*	*Buffer*	*Molybdate (M)*	*P_i liberated (µg/ml/16 min)*
1	β-Glycerophosphate	$2 \cdot 10^{-2}$	Citrate	—	20.4
	β-Glycerophosphate	$2 \cdot 10^{-2}$	Citrate	10^{-4}	20.8
	β-Glycerophosphate	$2 \cdot 10^{-2}$	Citrate	10^{-3}	19.6
2	β-Glycerophosphate	10^{-3}	Citrate	—	2.7
	β-Glycerophosphate	10^{-3}	Citrate	10^{-4}	3.0
3	β-Glycerophosphate	10^{-3}	Acetate	—	8.8*
	β-Glycerophosphate	10^{-3}	Acetate	$2 \cdot 10^{-4}$	0.3*
4	β-Glycerophosphate	10^{-3}	Acetate	—	9.0*
	β-Glycerophosphate	10^{-3}	Acetate	$2 \cdot 10^{-4}$	0.3*
	β-Glycerophosphate	10^{-3}	Acetate	$2 \cdot 10^{-4}$	0.3
5	TPP	10^{-3}	Acetate	—	11.2
	TPP	10^{-3}	Acetate	$2 \cdot 10^{-4}$	−0.3

* Yeast incubated with molybdate for 15 min before adding substrate.

out the next experiments in 0.1 *M* acetate (pH 3.7) and also pre-incubated yeast and molybdate. Indeed, the decomposition of β-glycerophosphate was enhanced in acetate as compared to citrate, and molybdate ($2 \cdot 10^{-4}$ *M*) now exerted a powerful inhibitory action (Table I, Expts. 3 and 4). The preincubation of yeast with molybdate proved to be quite unnecessary: the inhibition was just as strong when the yeast

TABLE II

INFLUENCE OF MOLYBDATE UPON THE DECOMPOSITION OF THIAMINE PYROPHOSPHATE BY ACID YEAST PHOSPHATASE IN DIFFERENT BUFFERS

Source of acid phosphatase: fresh baker's yeast 5 mg/ml. Buffers: 0.1 *M* citrate or 0.1 *M* acetate (pH 3.7). Reaction stopped by pipetting 1 ml of reaction mixture into 5 ml boiling 0.04 *N* HCl, containing 1 mg thiamine[10]. Further details as in Table I.

Expt. No.	*TPP (µg/ml)*	*Buffer*	*Molybdate (M)*	*TPP decomposed (µg/10 ml/4 min)*
1	2.5	Citrate	—	23.2
	2.5	Citrate	10^{-4}	22.2
	2.5	Citrate	10^{-3}	20.3
2	2.5	Acetate	—	19.6
	2.5	Acetate	10^{-4}	0.4

was added to a mixture already containing both substrate and molybdate (Table I, Expt. 4). Evidently, citrate ions prevent molybdate from displaying its inhibitory action.

Under the same circumstances the decomposition of TPP is also inhibited by molybdate; again this is not the case when citrate buffer is used (Table I, Expt. 5; Table II). Likewise, the acid phosphatase of dried brewer's yeast is inhibited by molybdate in acetate buffer of pH 3.7 (Table III, Expt. 1).

It became apparent from these experiments that the failure of molybdate to inhibit the acid phosphatase in the determination of TPP would be due to the employment of phosphate buffer. Phosphate ions do not themselves inhibit this

TABLE III

INHIBITION BY MOLYBDATE OF THE DECOMPOSITION OF THIAMINE PYROPHOSPHATE BY DRIED BREWER'S YEAST AT pH 3.7 AND 6.2

Source of acid phosphatase: dried brewer's yeast. Buffers: 0.1 *M* acetate (pH 3.7) or 0.1 *M* maleate (pH 6.2). Further details as in Table II.

Expt. No.	*TPP (µg/ml)*	*Yeast (mg/ml)*	*Buffer*	*pH*	*Molybdate (M)*	*TPP decomposed (µg/10 ml/4 min)*
1	2.5	10	Acetate	3.7	—	18.4
	2.5	10	Acetate	3.7	10^{-4}	0
2	2.5	50	Maleate	6.2	—	24.2
	2.5	50	Maleate	6.2	10^{-4}	5.6
	2.5	50	Maleate	6.2	10^{-3}	2.4

phosphatase[13], but they could perhaps prevent molybdate ions from doing so by forming a phosphomolybdate complex. So we looked for another buffer in the range of pH 6–7, and found maleate (0.1 *M*)[14] to be suitable. When the decomposition of small amounts of TPP is examined in 0.1 *M* maleate (pH 6.2), using the large amount of dried brewer's yeast that is also employed in the determination of TPP, 10^{-4} *M* molybdate does not afford full protection, but 10^{-3} *M* is satisfactory (Table III, Expt. 2).

The determination of TPP, using molybdate instead of thiamine as inhibitor of acid phosphatase

When the determination of TPP is carried out in 0.1 *M* maleate (pH 6.2) instead of in 0.1 *M* phosphate, still using thiamine as phosphatase inhibitor, the same high calibration curve is obtained (Fig. 1, Curves A and C). And as Table IV shows, equal amounts of CO_2 are produced from pyruvate when the alkaline-washed yeast is supplemented with molybdate (10^{-3} *M*) instead of thiamine (100 µg/ml yeast suspension), and both tests are run in 0.1 *M* maleate (pH 6.2).

Thus, when one wishes to avoid the use of thiamine in the determination of TPP, this can be replaced by molybdate if at the same time the phosphate buffer (pH 6.2) is replaced by maleate buffer of the same pH.

The prescription for the determination of TPP is accordingly modified as follows: Alkaline washing of the dried brewer's yeast is carried out in the usual manner[10] (in our case 5 min with 20 ml 0.1 *M* phosphate (pH 9.3)/g yeast at 16–18°, followed by 3 rapid washes with cold distilled water). The yeast is now suspended in 0.1 *M* maleate (pH 6.2) to a final volume of 10 ml/g of yeast, adding 1 ml 0.1 *M* $MnCl_2$ and 1 ml 0.01 *M* sodium molybdate. 0.5 ml of this yeast suspension is pipetted into the main compartment of each Warburg vessel, already containing 1 ml of TPP

TABLE IV

COMPARISON OF CO_2 PRODUCED FROM PYRUVATE BY ALKALINE-WASHED YEAST AND STANDARD AMOUNTS OF THIAMINE PYROPHOSPHATE WITH EITHER ADDED THIAMINE OR MOLYBDATE

Alkaline-washed dried brewer's yeast supplemented with 100 μg thiamine/ml (T) or 10^{-3} *M* molybdate (M); 0.5 ml suspension in each Warburg flask. Amounts of TPP (1 ml) per flask as indicated in the table. All runs in duplicate. Buffer: 0.1 *M* maleate (pH 6.2). Sodium pyruvate (2.5%): 0.2 ml in side-arm. Incubation at 28°. Further details in text.

Time (min)	*μl CO_2 produced by μg TPP*							
	0		*0.05*		*0.1*		*0.2*	
	T	*M*	*T*	*M*	*T*	*M*	*T*	*M*
10	2	0	22	21	36	38	61	63
20	6	0	44	43	77	74	119	122
30	7	3	65	67	110	109	171	174

solution. From this point on the determination proceeds as described previously[10].

Standard solutions of TPP are made up in 0.1 *M* maleate (pH 6.2). Unknowns are diluted with this buffer. Sodium pyruvate (2.5%) is also dissolved in it.

The concentration of the maleate buffer and the time at which TPP is added to the alkaline washed yeast

The apocarboxylase in the alkaline-washed yeast is extremely labile; so the sooner the latter is added to TPP after its preparation, the higher the amount of CO_2 produced from pyruvate .

In some experiments it is not possible to mix TPP and alkaline-washed yeast immediately, for example when OTPP is measured by its inhibitory action on the resynthesis of carboxylase[7]. The alkaline-washed yeast is then pre-incubated with OTPP and TPP is added 6 or 8 min later. In measurements of this kind we observed a fairly rapid loss of enzyme activity when the alkaline-washed yeast was incubated at 28° in 0.1 *M* maleate buffer (pH 6.2). This loss seemed to surpass that in 0.1 *M* phosphate under comparable circumstances. Further tests showed that the inactivation of apocarboxylase is much less in 0.02 *M* maleate than in 0.1 *M* maleate (Table V). So in certain cases it can be advantageous to carry out the determination of TPP in the less concentrated maleate buffer. When TPP and alkaline-washed yeast are mixed as soon as possible, the concentration of the buffer has very little influence upon the resulting enzyme activity, however.

TABLE V

INFLUENCE OF BUFFER CONCENTRATION AND TIME OF ADDITION OF THIAMINE PYROPHOSPHATE UPON RESYNTHESIS OF CARBOXYLASE

20 mg alkaline-washed brewer's yeast plus 10^{-3} *M* molybdate per Warburg flask. TPP: 0.2 μg. Buffers: maleate (pH 6.2) 0.1 *M* and 0.02 *M*. Total volume (including pyruvate): 1.7 ml. Incubation at 28°. I: added immediately to alkaline-washed yeast in main compartment. II: added to main compartment 6 min later from first side-arm. Pyruvate added 10 min after II, from second side-arm. $1/t_{60}$, reciprocal of time in which 60 μl CO_2 is produced.

Expt. No.	*Maleate (M)*	*I*	*II*	$1/t_{60} \times 100$	%
1	0.1	TPP	Buffer	10.6	100
	0.1	Buffer	TPP	7.2	68
2	0.02	TPP	Buffer	11.5	100
	0.02	Buffer	TPP	10.0	87

DISCUSSION

The experiments described above have shown that molybdate is indeed a powerful inhibitor of the acid yeast phosphatase, both in fresh baker's yeast and in dried brewer's yeast, provided the reaction mixtures are made up with a suitable buffer. In citrate or phosphate buffer no inhibition by molybdate is observed. The buffer ions (0.1 *M*) are always in large excess of the molybdate ions (10^{-3}–10^{-4} *M*), so in case of interaction or interference between the 2 kinds of ions it is evident that molybdate would have no chance for displaying any other kind of action. We presume that the citrate and phosphate ions interfere through complex formation, and that these complexes of molybdate no longer have an inhibitory action, perhaps because their shape or size prevents them from occupying a vital site on the surface of the phosphatase protein.

In a suitable buffer the inhibitory action of molybdate is instantaneously and fully developed; it is not necessary to pre-incubate yeast and molybdate before adding the phosphatase substrate, as ROTHSTEIN has recommended.

The manometric determination of TPP can be carried out in 0.1 *M* maleate buffer (pH 6.2) and with 10^{-3} *M* molybdate as inhibitor of acid phosphatase without any loss of sensitivity as compared to the usual procedure in 0.1 *M* phosphate buffer and with thiamine as phosphatase inhibitor.

In experiments not described in this paper, molybdate (10^{-3} *M*) has also proved to be an efficient inhibitor of the acid phosphatase present in partially-purified preparations of thiaminokinase. Using molybdate and working in 0.1 *M* maleate (pH 6.8) a good phosphorylation of thiamine by ATP is obtained with such enzyme preparations, even in the absence of phosphate, notwithstanding the fact that low concentrations of the latter compound greatly enhance thiaminokinase activity[8]. In principle it should be possible in this manner to examine whether yeast thiaminokinase can also synthesize OTPP from oxythiamine and ATP. This question is of interest because there are reasons for presuming that oxythiamine will mainly exert its action as antagonist of thiamine by competing with TPP after pyrophosphorylation[7]. However, still other difficulties present themselves here, mostly arising from the lack of a sensitive method for determining OTPP.

It may be mentioned here that ATP proved to have a small but definite stimulative action (about 10%) on the resynthesis of carboxylase, if added to the alkaline-washed yeast before adding TPP, in a concentration of 1 μmole per Warburg flask. Probably this action is not a protection of the apocarboxylase, as the addition of ATP does not prevent the apoenzyme from deteriorating with time, and higher concentrations of ATP are inhibitory. On the other hand, ATP itself is decomposed fairly rapidly by the alkaline-washed dried yeast: in a concentration of 1.5 μmole ATP/ml about half is hydrolysed in 8 min at pH 6.2, 2 phosphate groups being split off. 10^{-3} M molybdate does not prevent this hydrolysis. At the present we have no satisfactory explanation for this action of ATP on carboxylase resynthesis.

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