THIAMINE-PYRAMIN ASSAY

Factors Influencing the Yeast Fermentation Methods for Thiamine and Pyramin

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In a study of the yeast fermentation methods for thiamine and pyramin, a number of physical and chemical factors were found to affect the analytical result. A new and more sensitive criterion for the adequacy of shaking was developed. Sources of interlaboratory variation in the determination of the sulfite blank activity are discussed. New limitations in the conditions of the AOAC thiamine method will increase the reliability of the method. As pyramin is the metabolic breakdown product of thiamine which is normally excreted in human urine, the accurate determination of both thiamine and pyramin is of considerable importance in the nutritional and clinical study of vitamin B_1 metabolism.

THIAMINE AND CERTAIN PYRIMIDINES related to it have a stimulatory effect upon the rate of yeast fermentation. Sulfite destroys this activity of thiamine but is without effect upon the activity of certain pyrimidines chemically related to it. This is the basis of the thiamine assay of Schultz, Atkin, and Frey (*14*), which has been accepted with minor modifications (*10*) as an official AOAC method for thiamine (*1*).

The procedure involves the measurement of the carbon dioxide evolved by yeast suspended in a nutrient medium to which has been added (*a*) thiamine standards, (*b*) an aliquot of the assay sample, or (*c*) an aliquot of the sulfited assay sample. The result obtained from (*b*) is termed the "total" fermentation activity, and is considered to be a measure of both thiamine and its pyrimidine derivatives. The result from (*c*) is termed the "residual" or "sulfite blank" activity, and is attributed to the active pyrimidines. The difference between the total and residual activities is termed the "true" thiamine activity. It is from a comparison of this true thiamine difference with the thiamine standard data obtained in (*a*) that the thiamine concentration in the original sample is estimated.

Pyrimidine accelerating yeast fermentation (PAYF) is a general term that has been applied (*6*) to both the natural and synthetic substances which yield this residual or sulfite blank activity. Of the synthetic pyrimidines that are able to increase the rate of yeast fermentation, 2-methyl-4-amino-5-ethoxymethylpyrimidine, an intermediate in the commercial synthesis of thiamine, is readily available in pure crystalline form and has been most widely used (*3, 4, 6–8, 12, 14, 15*). Several other synthetic pyrimidines have been reported to have approximately the same activity (*8*). The number of active pyrimidines occurring in nature is yet to be established. Pyramin, the pyrimidine activity in human urine (*12*), is a metabolic breakdown product of thiamine (*3, 12, 13*) and is thus of special nutritional and clinical interest. It may be distinguished from certain active pyrimidines occurring in food products by its stability to heating in acid solution (*3*).

The determination of pyramin (*4*) involves the comparison of the fermentation stimulation produced by sulfited urine with that produced by known standards of 2-methyl-4-amino-5-ethoxymethylpyrimidine. Pyramin values are calculated and reported in terms of this standard, rather than in terms of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride, as previously stated (*3, 4, 12*).

The analytical problems in the determination of pyramin are essentially those of determining the residual or sulfite blank activity in the thiamine method. Because the yeast fermentation methods for thiamine (*1, 14*) and pyramin (*4*) have so much in common, many of the physical and chemical factors affecting one are also of importance in the other.

Sulfiting Errors

From the results of an AOAC collaborative assay, Kline (*10*) concludes that sulfiting is a critical and difficult step which needs further study.

The authors have studied two variables in the sulfiting procedure—boiling time and sulfite-peroxide end point—as possible sources of error. Boiling times ranging from 30 to 120 minutes were without effect upon the fermentation activity of pyramin. However, this factor may still be of importance in the thiamine method, particularly when it is applied to the analysis of food which has been heat-processed or stored. Caster and Mickelsen (*3*) have demonstrated that the fermentation activity of certain degradation products of thiamine is easily destroyed by heating in acidic solutions. Under such circumstances, slight differences in the handling of the assay sample and sulfite blank might well produce small but consistent differences in thiamine values obtained from samples containing these labile pyrimidines.

In Figure 1 it is seen that the sulfite-peroxide end point is critical, particularly on the side of adding excess peroxide. This could easily be a source of substantial error. The sulfiting directions given by Kline (*1, 10*) state that a change of acidic starch-iodide indicator color from pink to blue indicates a complete destruction of sulfite. The use of this criterion for determining the end point might lead to an error in the direc-

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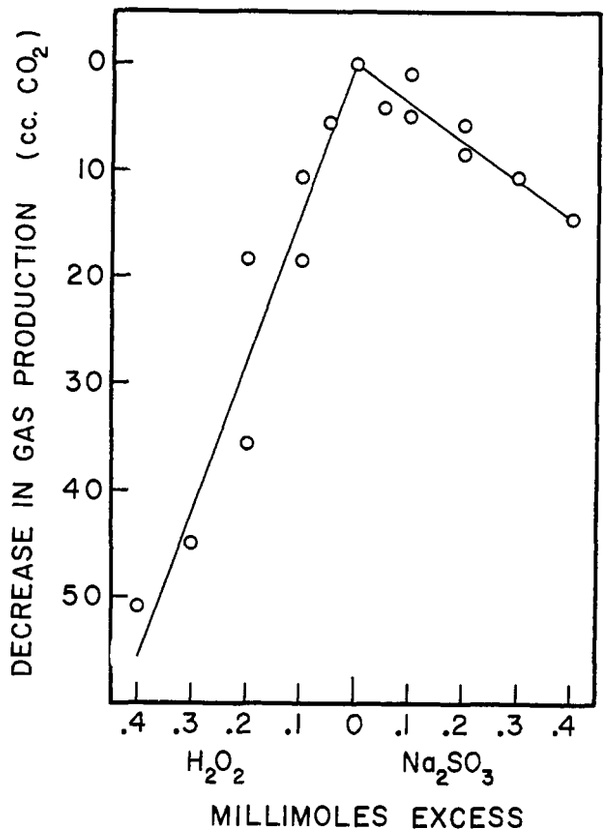


Figure 1. Sulfite-peroxide end point

An excess of hydrogen peroxide or sodium sulfite was added to fermentation vessels containing 2-methyl-4-amino-5-ethoxymethylpyrimidine

tion of adding a slight excess of peroxide. A small excess of peroxide is relatively more inhibitory, and thus more to be avoided, than an equal excess of sulfite. The sulfiting procedure in the pyramin method (*4*) is designed specifically to avoid this peroxide error.

Fermentation Rate Studies

The rate with which gas is evolved throughout the period of fermentation has been studied as a means of determining the relation between thiamine activity and the sulfite blank activity. The results in Figure 2 are typical. Very little differentiation appears even between the blank and the highest standards within the first hour. In Figure 2, the thiamine and pyrimidine standards are present in approximately equimolar quantities, while the 4-methyl-5-β-hydroxyethylthiazole is present in excess.

The gas evolution curves of equimolar standards of thiamine and 2-methyl-4-amino-5-ethoxymethylpyrimidine are not identical. What is perhaps more important is that in their linear portions—i.e., after 2.0 to 2.5 hours—they are not parallel. In other words, the ratio of thiamine activity to nonthiamine activity is dependent upon the length of the fermentation period. The longer the fermentation period, the larger is the pyrimidine blank activity as a percentage of total activity. This fact calls into question those fermentation methods with variable fermentation periods. The AOAC procedure (*1*) allows the fermentation period to range from 3 to 3.5 hours in length. This feature of the method may furnish a further explanation of interlaboratory discrepancies in the estimation of sulfite blank activity reported by Kline (*10*).

Activity of Sulfiting Products

During the sulfiting process 1 mole of 4-methyl-5-β-hydroxyethylthiazole is produced for each mole of thiamine destroyed in the sulfiting procedure. This thiazole compound is reported (*5*) to have a slight activity in the fermentation. The authors' data confirm this, but the activity is very small.

An additional action of the thiazole is demonstrated in Figure 2. 4-Methyl-5-β-hydroxyethylthiazole has a marked effect in increasing the activity of the 2-methyl-4-amino-5-ethoxymethylpyrimidine but does not affect thiamine. This action occurs through a decrease in the induction period of the fermentation (which causes it to correspond more nearly with that observed for thiamine) but without appreciably changing the final slope of the pyrimidine gas evolution curve. Thus the thiazole moiety of thiamine, though by itself only slightly active, may be a significant source of error in the fermentation procedure for the determination of both pyramin and thiamine in high blank materials.

Deutsch (*5*) reports that the 2-methyl-4-amino-5-sulfomethylpyrimidine produced by the sulfite reaction is also active in the fermentation procedure. To check this point, this sulfonic acid was prepared from thiamine by the method of Williams *et al.* (*16*) and recrystallized three times, and its fermentation activity was tested. This compound was completely inactive in amounts as large as 100 γ per flask. It is probable that the activity noted by Deutsch was due to a trace of thiamine or thiazole present as an impurity in his product.

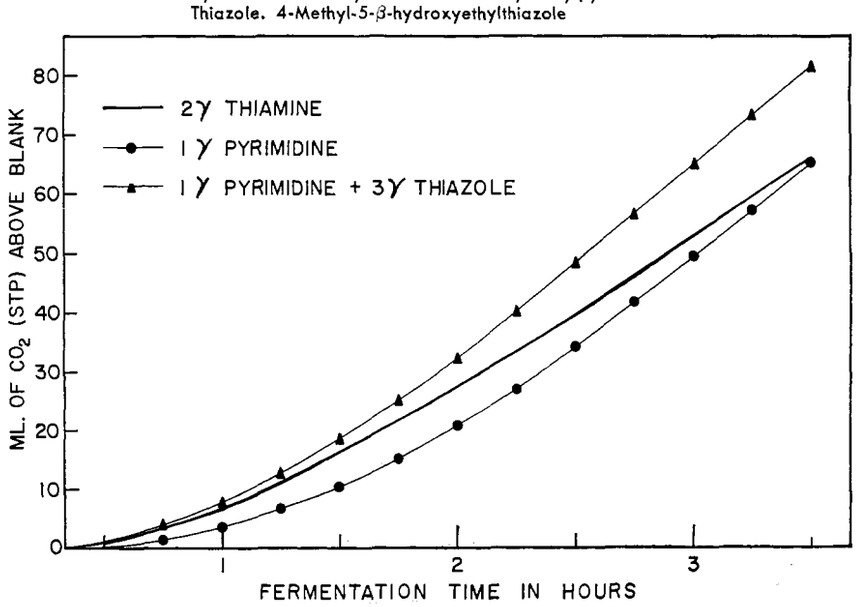
Additivity of Thiamine And Pyrimidine

One of the basic assumptions in the yeast fermentation method for thiamine is that the activities of thiamine and the pyrimidine blank materials are additive. Such an assumption is required before one can subtract the residual sulfite blank activity from the total fermentation activity to obtain a true thiamine activity as a difference.

Figure 3 shows how far from true this assumption can be in the case of 2-methyl-4-amino-5-ethoxymethyl-

Figure 2. Gas evolution curves

Pyrimidine. 2-Methyl-4-amino-5-ethoxymethylpyrimidine
Thiazole. 4-Methyl-5-β-hydroxyethylthiazole



pyrimidine. Here the carbon dioxide produced by thiamine standards, and various mixtures of thiamine and pyrimidines, is compared with the gas volumes produced by pyrimidine standards that are their molar equivalents. If one assumes that the fermentation activities of thiamine and pyrimidine are additive, all the lines in Figure 3 should be straight. For mixtures which are the molar equivalent of 2 γ of thiamine this is approximately true. At lower and particularly at higher total activities, substantial errors may be involved.

It is usually suggested that when the sulfite blank is small, 1.0 γ of thiamine be added to raise the observed activity, so that it is equivalent to that of 1 to 2 γ of thiamine. The present data emphasize the even greater importance of limiting the upper activities that are allowable in this procedure.

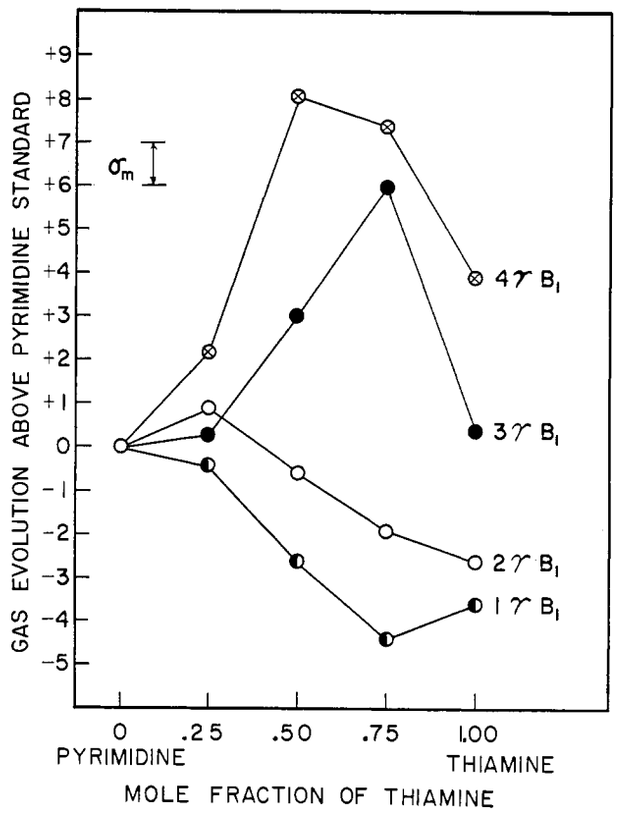


Figure 3. Additivity of pyrimidine and thiamine

Pyrimidine standard was 2-methyl-4-amino-5-ethoxymethylpyrimidine. Gas evolution is given in cc. of CO_2 above the blank. For each level of thiamine studied, appropriate amounts of pyrimidine and thiamine were added to provide the indicated molar fractions

Adequacy of Shaking

The fermentometer used (*4*) was originally designed to provide a shaking rate of 96 excursions per minute as suggested by Schultz, Atkin, and Frey (*14*). Under these conditions, marked position differences were observed within the fermentometer (see Figure 4). One method of dealing with this difficulty would be to introduce position corrections for the different fermentation units. A more satisfactory method, however, was provided by increasing the intensity or speed, of shaking.

A flexible coupling introduced into the shaking mechanism resulted in a much more vigorous shaking motion without any increase in speed of shaking. The position factors immediately disappeared. The flexible coupling was noisy and showed signs of rapid wear. It was removed, and the gears were changed to provide a shaking rate of 138 revolutions per minute. Position corrections were also absent under these conditions.

None of these changes had any measurable effect upon the shape or height of the yeast fermentation response curve. However, there was a marked change in the analytical precision of this procedure, as seen in Table I. Increasing the shaking not only eliminated the position - to - position corrections within the fermentometer but also virtually eliminated the differences observed between consecutive runs in the fermentometer. This had the effect of reducing the total manipulative error to less than half its previous level.

It is frequently considered that the attainment of maximal dose-response curve is proof of the adequacy of shaking in a system such as this. It appears from these data, however, that the elimination of consistent position-correction factors and between-run discrepancies constitutes a more sensitive indicator of the adequacy of shaking. Specifying speed and length of stroke does not provide an adequate index of the vigor of agitation.

Discussion

The effect of a number of physical and chemical factors upon the yeast fermentation method for thiamine and pyramin has been studied. Among the potential sources of error in the pyramin method are the effects resulting from addition of excess peroxide in the sulfiting step, inadequate shaking during the fermentation period, and the stimulatory action of 4 - methyl - 5 - β - hydroxyethylthiazole. Physiological and dietary factors have been discussed (*4*).

Some thought was given to the addition of an excess of 4 - methyl - 5 - β-hydroxyethylthiazole to the fermentation medium used for the determination of pyramin (*4*). It was found, however, that the addition of this thiazole to sulfited urine had little or no effect upon the observed pyramin activity. Also, the induction period in the fermentation of pyramin corresponds more nearly with thiamine than with synthetic pyrimidine. These two findings may indicate that sulfited urine samples already contain effective amounts of this compound. Part of this thiazole is normally present in urine (*2*, *9*), and additional amounts are produced by the sulfiting procedure.

A number of additional factors may furnish problems in the yeast fermentation method for thiamine: the presence, in certain foods, of a mixture of active pyrimidines with differing degrees of stability, the nonadditivity of the activities of thiamine and the active pyrimidine tested, and the dependence of the observed thiamine to nonthiamine activity ratio upon the length of the fermentation period. Each of these factors assumes importance in direct proportion to the relative size of the sulfite blank activity.

In the AOAC collaborative assay for thiamine (*10*), the sulfite blank, or residual activity, of the flour, bread, and yeast samples averaged, respectively, 16, 21, and 21% of the total activity. A statistical analysis of these data (*11*) is summarized in Table II. In spite of its small size, this residual activity accounts for nearly as much—in one case more—variation than the total activity.

The fact that $\sqrt{\sigma^2_{total} + \sigma^2_{residual}}$ does not equal or even approximate σ_{true} in several cases, indicates an interaction between the observed thiamine and sulfite blank activities. This is particularly true in the bread and yeast samples, and may point to the presence of heat- or sulfite-labile pyrimidine in these samples. Autoclaved yeast extract has been shown to contain such a heat-labile pyrimidine (*4*).

The relatively larger variation associated with the determination of sulfite blank activity, the nonadditivity of thiamine and pyrimidine activities, and the presence of labile pyrimidines, all indicate the advisability of limiting the use of this thiamine method to those

Table I. Effect of Shaking Rate upon Variation in Fermentation Results

(Expressed in terms of gas volumes)

	Standard Deviations, Cc.	
Source of Variation	*96 r.p.m.*	*138 r.p.m.*
Random	1.05	1.05
Position to position	1.24	0
Between runs	1.98	0.33
Total	2.56	1.12

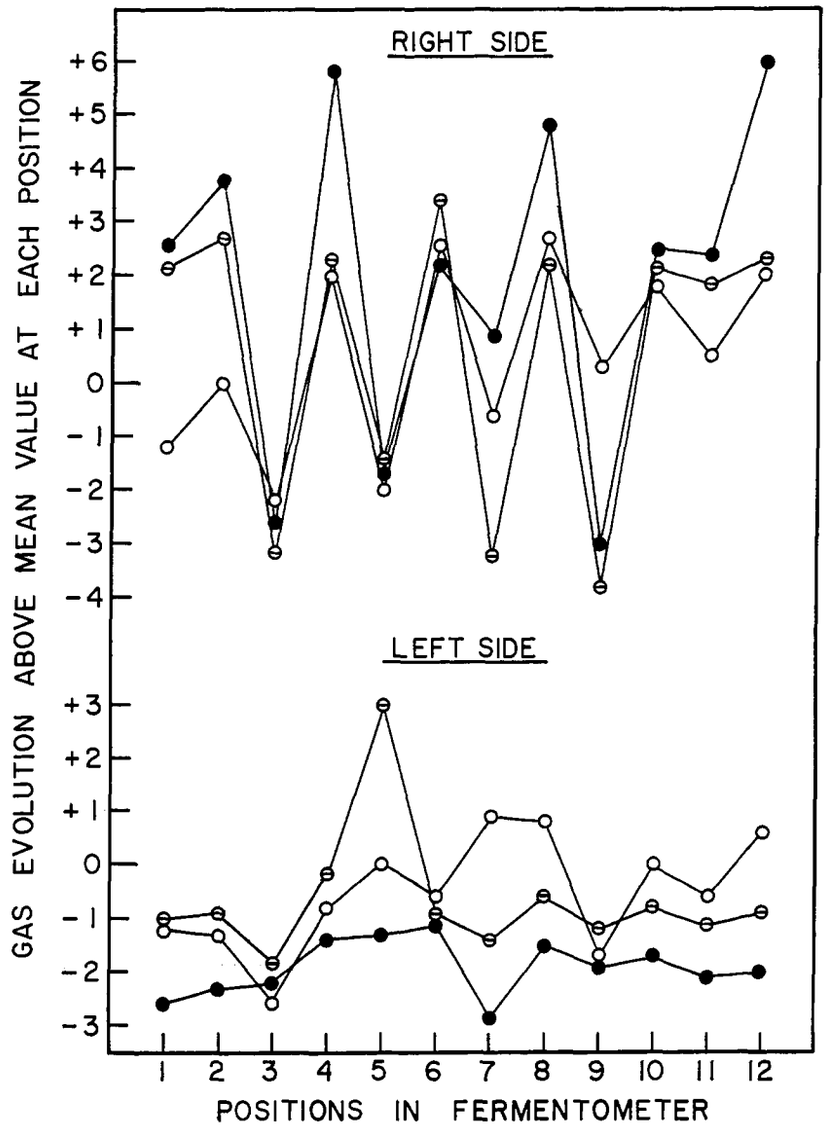


Figure 4. Position corrections

Deviations in gas evolution from mean value are given for three separate trials. Same amount of pyrimidine standard was used in each fermentation vessel

samples which have relatively low sulfite blank activities.

Schultz, Atkin, and Frey (*14*) report that in most of the different types of samples that they tested, the sulfite blank activity was 10% or less of the total activity. They do not propose any upper limit on the relative size of the sulfite blank. The AOAC comes somewhat closer to setting an upper limit in blank activity when it specifes that, in choosing aliquots, the assay sample should contain an estimated 1.5 γ of thiamine, and the total activity should not exceed 2.0 γ of thiamine (*1*). Taken literally, this would limit the sulfite blank to a maximum of 25% of the total activity. Such a limitation would not seriously limit the utility of the method, but would rather increase its reliability. However, the AOAC procedure goes on to provide (*1*, Section 40.27) that if the sulfite blank activity exceeds that of the 1 γ thiamine standard, the usual addition of 1.0 γ of thiamine to the blank be omitted. This implies that high blank materials may also be used. In view of above observations, a statement clearly limiting use of this method to low blank materials would seem more appropriate.

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Table II. Within-Laboratory (σ_{wl}) and Between-Laboratory (σ_l) Standard Deviations Calculated from Thiamine Assay Data (*11*)

(Expressed as per cent of mean true value in each case)

	Flour		Bread		Yeast	
	σ_{wl}	σ_l	σ_{wl}	σ_l	σ_{wl}	σ_l
Total	5.72	7.75	7.76	11.12	6.54	6.76
Residual	5.73	9.13	6.61	7.37	4.55	5.76
True	7.12	15.36	7.07	9.86	6.08	4.70