EXPERIMENTS ON THE FORMATION OF CARBOXYLASE AND THIAMINE PYROPHOSPHATE IN LIVING BAKERS' YEAST*

by

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INTRODUCTION

SPERBER AND RENVALL[1] have found that thiamine, added to fresh yeast, is converted into thiamine pyrophosphate. The latter compound is the prosthetic group of carboxylase, the enzyme responsible for the splitting of pyruvic acid into acetaldehyde and CO_2. WESTENBRINK, STEYN-PARVÉ AND VELDMAN[2], while confirming these results, further concluded from their experiments that the increase of thiamine pyrophosphate was not accompanied by any increase of carboxylase.

However, according to ATKIN, SCHULTZ AND FREY[3] the rate of CO_2 production from glucose by living yeast is considerably increased by adding small amounts of thiamine. In the course of investigations on the mode of action of the thiamine in this activation of fermentation it became imperative to reinvestigate the problem, whether any carboxylase was formed from the added thiamine or not. For WESTENBRINK *et al.* had indeed demonstrated without any doubt, that the great bulk of thiamine pyrophosphate, formed from added thiamine, was not present as the prosthetic group of carboxylase, but their method of analysis did not exclude the possibility that a small increase of the carboxylase content might have escaped observation. At the request of Prof. WESTENBRINK we have therefore once more studied the formation of carboxylase from added thiamine by fresh bakers' yeast, but now with a method of determination which entails less risk of decomposition of small amounts of carboxylase.

The carboxylase content of yeast, expressed as the pyruvate decarboxylating activity of yeast, can only be determined after the yeast has been rendered permeable to added pyruvate. This can either be done by drying the yeast at room temperature or by repeated freezing and thawing. WESTENBRINK *et al.* have employed the former of these procedures, but it was deemed possible that part of the carboxylase might have been lost during the drying. Therefore we have now used the latter method, a procedure in which the carboxylase certainly runs little risk of destruction, and we have indeed found that under certain conditions, notably in the presence of added thiamine or

* This work forms part of the investigations on the action and metabolism of thiamine in yeast by H. G. K. WESTENBRINK and collaborators. Requests for reprints should be addressed to Professor WESTENBRINK.

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2-methyl-4-amino-5-ethoxymethylpyrimidine, NH_4-ions and glucose, carboxylase is formed.

As the question was studied in view of the explanation of the effect of thiamine on fermentation, as discovered by ATKIN, SCHULTZ, AND FREY[3], the concentrations of the reaction components were similar to those usual in the determination of thiamine based upon this effect.

EXPERIMENTAL PART

Methods

The reaction mixtures always had a volume of 100 ml. They were placed in 250 ml Erlenmeyer flasks and shaken for 4 hours under nitrogen (not strictly anaerobically) in a bath of 27° C. They always contained 200 mg fresh bakers' yeast ("Koningsgist", Delft); the medium was 0.1 *M* acetate buffer, p_H 5.6. When present, the concentration of glucose was 2.4%, the concentration of NH_4 sulfate 0.1%, the concentration of thiamine 6 γ per 100 ml and the concentration of "pyrimidyl" (2-methyl-4-amino-5-ethoxymethylpyrimidine) 3 γ per 100 ml.

After incubation the yeast was spun off in nickel-plated centrifuge tubes and washed with 0.1 *M* acetate buffer, p_H 5.6. The tubes containing the sedimented yeast were then placed in a mixture of acetone and solid carbon dioxide for 15 minutes. The frozen yeast was then rapidly thawed by holding the tubes under running tap water. This procedure of freezing and thawing was repeated four times. (When the freezing and thawing were carried out only once very bad duplicates were obtained; obviously it is necessary to repeat the procedure in order to affect all cells).

Carboxylase was determined as follows: 0.1 *M* acetate buffer, p_H 5.6, was added, making up the volume to 25 ml. 1.5 ml of this suspension was placed in the main compartment of a Warburg flask and 5 mg Na pyruvate, dissolved in 0.1 *M* acetate buffer, p_H 5.6, in the side bulb. The production of carbon dioxide was measured at 27° C for 30 minutes after tipping in the pyruvate. The manometers were read every 10 minutes.

Thiamine pyrophosphate was determined according to WESTENBRINK AND STEYN-PARVÉ[4].

RESULTS

The carboxylase formation in the various reaction mixtures as expressed by the CO_2 production from Na pyruvate in the first 10 minutes under the influence of 1.5 ml of the suspension of yeast rendered permeable by alternative freezing and thawing, can be seen from Tables I to IV. It is evident that carboxylase is formed upon incubation of the yeast with thiamine or "pyrimidyl" (2-methyl-4-amino-5-ethoxymethylpyrimidine) as well in the absence as in the presence of glucose. The effect of thiamine seems to be a little stronger than that of "pyrimidyl". Only very little carboxylase if any, is formed upon incubation with NH_4 sulfate only. In the presence of glucose, however, NH_4 sulfate induces carboxylase formation, not less than either caused by thiamine or "pyrimidyl". In the absence of glucose the effect of thiamine + NH_4 sulfate or pyrimidyl + NH_4 sulfate is not significantly higher than the effect of thiamine or "pyrimidyl" alone. In the presence of glucose, however, the effects of thiamine + NH_4 sulfate or "pyrimidyl" + NH_4 sulfate considerably surpass the effect of any of these substances separately.

The effect of "pyrimidyl" can be explained by the primary formation of thiamine pyrophosphate from "pyrimidyl". Indeed in two experiments we found that the yeast contained 19.0 γ and 18.4 γ thiamine pyrophosphate per g after incubation in the absence of added "pyrimidyl" and 27.2 γ and 32.0 γ respectively after incubation in the presence of this compound. This is in contradiction to the observation of FINK AND JUST[5], who also investigated bakers' yeast and did not find thiamine or thiamine pyrophosphate synthesis from added 2-methyl-4-amino-5-hydroxymethylpyrimidine.

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The effect of NH_4 sulfate in the presence of glucose may be explained by the synthesis of apocarboxylase and displacement of thiamine pyrophosphate from other bearers to this protein. The optimal effect of thiamine or "pyrimidyl" together with NH_4 sulfate in the presence of glucose obviously depends upon the synthesis of both apocarboxylase and thiamine pyrophosphate.

TABLE I

INFLUENCE OF THIAMINE, NH_4 SULFATE, AND THIAMINE + NH_4 SULFATE ON THE FORMATION OF CARBOXYLASE BY BAKERS' YEAST

GLUCOSE ABSENT

Additions	mm^3 CO_2 in 10 minutes		
	1st exp.	2nd exp.	3rd exp.
—	102	109	90
Thiamine	125	131	104
NH_4 sulfate	100	104	95
Thiamine + NH_4 sulfate	123	126	109

TABLE II

INFLUENCE OF "PYRIMIDYL", NH_4 SULFATE, AND "PYRIMIDYL" + NH_4 SULFATE ON THE FORMATION OF CARBOXYLASE BY BAKERS' YEAST

GLUCOSE ABSENT

Additions	mm^3 CO_2 in 10 minutes				
	1st exp.	2nd exp.	3rd exp.	4th exp.	5th exp.
—	102	102	90	95	90
"Pyrimidyl"	113	104	104	109	100
NH_4 sulfate	104	109	99	101	96
"Pyrimidyl" + NH_4 sulfate	116	113	109	116	106

TABLE III

INFLUENCE OF THIAMINE, NH_4 SULFATE, AND THIAMINE + NH_4 SULFATE ON THE FORMATION OF CARBOXYLASE BY BAKERS' YEAST

GLUCOSE PRESENT

Additions	mm^3 CO_2 in 10 minutes			
	1st exp.	2nd exp.	3rd exp.	4th exp.
—	89	94	108	80
Thiamine	107	113	129	106
NH_4 sulfate	112	118	133	—
Thiamine + NH_4 sulfate	179	184	207	—

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TABLE IV

INFLUENCE OF "PYRIMIDYL", NH_4 SULFATE, AND "PYRIMIDYL" + NH_4 SULFATE ON THE FORMATION OF CARBOXYLASE BY BAKERS' YEAST

GLUCOSE PRESENT

Additions	mm^3 CO_2 in 10 minutes			
	1st exp.	2nd exp.	3rd exp.	4th exp.
—	111	95	97	101
"Pyrimidyl"	126	112	109	113
NH_4 sulfate	133	117	—	—
"Pyrimidyl" + NH_4 sulfate	171	154	—	—

SUMMARY

The formation of carboxylase by living bakers' yeast was demonstrated upon incubation of the yeast with either thiamine or 2-methyl-4-amino-5-ethoxymethylpyrimidine, in the presence and in the absence of glucose. Carboxylase is also formed upon incubation of the yeast with NH_4 sulfate and glucose. In the presence of glucose the effects of thiamine + NH_4 sulfate or 2-methyl-4-amino-5-ethoxymethylpyrimidine + NH_4 sulfate considerably surpass the effects of any of these substances separately.

The effect of 2-methyl-4-amino-5-ethoxymethylpyrimidine depends upon the primary formation of thiamine pyrophosphate.

RÉSUMÉ

La formation de carboxylase par de la levure des boulangers vivante a été démontrée par incubation de la levure soit avec de la thiamine soit avec de la 2-méthyl-4-amino-5-éthoxyméthyl pyrimidine en présence et en absence de glucose. La formation de carboxylase a lieu également lorsque la levure est incubée avec du sulfate d'ammonium et du glucose. En présence de glucose les effets de thiamine + sulfate d'ammonium ou de 2-méthyl-4-amino-5-éthoxyméthyl pyrimidine + sulfate d'ammonium dépassent considérablement les effets d'une quelconque de ces substances prise à part.

L'effet de la 2-méthyl-4-amino-5-éthoxyméthyl pyrimidine dépend de la formation primaire de pyrophosphate de thiamine.

ZUSAMMENFASSUNG

Die Bildung von Carboxylase durch lebende Bäckerhefe wurde durch Inkubation der Hefe mit Thiamin oder 2-Methyl-4-amino-5-äthoxymethyl-pyrimidin in Gegenwart und in Abwesenheit von Glukose bewiesen. Carboxylase wird auch bei Inkubation von Hefe mit Ammoniumsulfat und Glukose gebildet. In Gegenwart von Glukose übertrifft die Wirkung von Thiamin + Ammoniumsulfat oder 2-Methyl-4-amino-5-äthoxymethyl-pyrimidin + Ammoniumsulfat bedeutend die Wirkung irgendeiner dieser Substanzen allein.

Die Wirkung von 2-Methyl-4-amino-5-äthoxymethyl-pyrimidin hängt von der primären Bildung von Thiaminpyrophosphat ab.

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