50. THE EFFECT OF THIAMINE (VITAMIN B₁) ON FERMENTATION OF YEAST

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THIAMINE (vitamin B₁) is known to affect the metabolism and growth of yeast. It was found to stimulate the growth in concentrations as low as 0.01 $\mu\text{g./ml.}$ of culture medium [Williams & Roehm, 1930]. Not every type of yeast, however, is affected in this way, some yeasts responding only very little to thiamine under the usual growth conditions. Similarly some yeasts only are capable of synthesizing thiamine from comparatively simple media [for lit. see Williams, 1941]. Closely related to the effect of thiamine on growth is its effect on yeast fermentation. This is increased by doses of thiamine of the same order of magnitude as those which stimulate growth [Schultz *et al.* 1937, 1, 2]. A quantitative test for determining such minute amounts by the yeast fermentation method has been described by Atkin *et al.* [1939] who found that the addition of 0.01–0.04 $\mu\text{g.}$ of thiamine per 5 mg. yeast (wet weight) suspended in 3 ml. of medium considerably raises the anaerobic CO₂ output during the 2nd hr. of incubation, and that the increase of fermentation caused by doses of 0.01 to 0.02 $\mu\text{g.}$ is proportional to the amount of added thiamine.

In this paper the experiments described by Atkin *et al.* [1939] have been repeated and some more facts have been gained on the effect of thiamine on yeast fermentation.

EXPERIMENTAL

The experiments were made with ordinary Warburg manometers (conical flasks of about 18 ml.) at 20 and 39°. N₂ was freed from traces of O₂ by passing it over heated copper.

For each vessel 1–5 mg. wet weight of ordinary baker's yeast or *Torula utilis* were used, suspended in the medium employed by Atkins *et al.* Their experimental procedure was generally followed except that the volume of fluid per vessel was always kept constant when different amounts of thiamine were tested (*vide* Table 1). The percentage increase in the rate of fermentation caused by thiamine above that of the control to which no thiamine had been added was plotted against concentration of thiamine.

Table 1

Exp. 12, Fig. 4. Period, 60–110 min. Anaerobic fermentation, 3 mg. yeast per vessel, 20°. Thiamine solution, 0.04 $\mu\text{g./ml.}$ Thiamine test solutions: A, 0.02 $\mu\text{g./ml.}$ B, 0.03 $\mu\text{g./ml.}$

	I	II	III	IV	V
Distilled water	2.0	1.0	1.75	1.0	1.0
Thiamine solution	—	1.0	0.25	1.0A	1.0B
Yeast susp.	1.0	1.0	1.0	1.0	1.0
<i>h</i> (mm.)	+41.0	+81.0	+63.75	+74.0	78.75
<i>k</i> _{CO₂}	1.887	1.893	1.903	1.801	1.823
$\mu\text{l. CO}_2$	+77.5	+153	+121	+133	+143
% increase above I	—	+97.8 %	+56.5 %	+71.9 %	+85.5 %

(488)

Results

Anaerobic fermentation of yeast. Fig. 1 shows the percentage increase of anaerobic fermentation at 20° of 5 mg. yeast per vessel for different periods from 0 to 150 min. induced by thiamine in concentrations of 0.01, 0.02 and 0.04 μg . per vessel. The experiment shows:

(1) There is a very marked increase of anaerobic fermentation under the influence of thiamine.

(2) The percentage increase is in definite relation to the amount of thiamine added.

(3) The absolute amount of CO_2 produced under the influence of thiamine increases with time, the relative percentage increase caused by different thiamine concentrations, however, remains more or less the same for each period.

Fig. 1.

Fig. 2.

Fig. 1. Percentage increase of anaerobic fermentation for different periods of the experiment. 5 mg. yeast (wet wt.) per vessel; 20°.

Fig. 2. Percentage increase of anaerobic fermentation for different periods of the experiment. 2 mg. yeast; 39°.

This last fact probably explains why Atkin *et al.* have used the 2nd hour for their determinations, for it is only in this period that the absolute increase of fermentation caused by a given amount of thiamine is significant enough to reveal comparatively small differences of the fermentation rate caused by different amounts of thiamine.

Fig. 2 shows the percentage increase of anaerobic fermentation by 2 mg. yeast per vessel for different periods from 0 to 72 min. with 0.01, 0.02 and 0.04 μg . thiamine at 39°. On the whole, this experiment closely resembles that of Fig. 1, the same relative increase of fermentation being caused by various amounts of thiamine, and the rate of fermentation increasing with time. The only difference from Fig. 1 is that all the curves lie on a slightly higher level. This represents the

temperature coefficient of the effect, which is evidently low, considering that the temperature difference is about 20° . The higher level of fermentation rate in this experiment does not seem to be caused by the fact that the same amount of thiamine is allowed to act on less yeast per vessel (2 mg. instead of 5 mg.) because the same thiamine concentration acting on half the amount of yeast (1 mg., Fig. 3) but at the same temperature (39°) does not further increase the rate of fermentation. Fig. 2 shows also that the quantitative testing of thiamine need not be restricted to the narrow range between 0.01 and 0.02 $\mu\text{g.}$ where the increase of fermentation is proportional to the amount of added thiamine. If the rates of fermentation are plotted against concentration of thiamine for different consecutive periods of the experiment a similar type of curve is obtained, which allows testing for thiamine over a wider range.

Fig. 3.

Fig. 3. Determination of the thiamine contents of two test solutions, $A=0.02 \mu\text{g./1.0 ml.}$, $B=0.03 \mu\text{g./1.0 ml.}$, by the percentage increase of anaerobic fermentation. 1 mg. yeast; 39° .

Fig. 4.

Fig. 4. Same as Fig. 3. 3 mg. yeast; 20° .

Fig. 3 represents the results of such an experiment, in which the strength of two thiamine solutions (A and B) was determined. Solution A , containing 0.02 mg./1.0 ml. , and B , containing $0.03 \mu\text{g./1.0 ml.}$, had been given to me without my knowing their thiamine contents. Two consecutive determinations of the fermentation rates were made for the periods 60–90 min. and 60–120 min. with 0.01 and 0.04 $\mu\text{g.}$ thiamine each. The thiamine contents of solutions A and B were determined by the fermentation rate of the same amounts of yeast for the period 60–120 min. The figure shows that the rates found for A and B correspond exactly to the amounts of thiamine present, i.e. 0.02 and 0.03 $\mu\text{g.}$

Fig. 4 (3 mg. yeast; 20°) shows a similar experiment where for the period 60–110 min. the fermentation rate was measured with 0.01 and 0.04 $\mu\text{g.}$ as standard and A (0.02 $\mu\text{g.}$) and B (0.03 $\mu\text{g.}$) as unknown thiamine concentra-

tions. Irrespectively of whether the points marking the fermentation rate with 0.01 and 0.04 $\mu\text{g.}$ were connected by a curve or a straight line, the values obtained for A and B lie close enough to either of them to allow the respective thiamine contents to be determined sufficiently accurately for all practical purposes.

Aerobic fermentation of yeast. This is quantitatively affected by thiamine in the same way as anaerobic fermentation, but the test can, of course, be made only with yeast varieties which normally have some aerobic fermentation. An example is shown in Fig. 5, where the percentage increase of aerobic fermentation is determined with 0.01, 0.02, 0.03 and 0.04 $\mu\text{g.}$ thiamine, the points marking the fermentation rates with 0.02 and 0.03 $\mu\text{g.}$ lying close to either a curve or a straight line connecting the points which correspond to the fermentation rate with 0.01 and 0.04 $\mu\text{g.}$ of thiamine.

Fig. 5. Determination of the thiamine contents of two test solutions, $A=0.02 \mu\text{g./ml.}$, $B=0.03 \mu\text{g./ml.}$ by the percentage increase of aerobic fermentation. 3 mg. yeast; 20° .

Technically the measurement of aerobic fermentation is simpler and quicker than that of anaerobic fermentation. It requires, however, in addition to the usual manometer (I), another manometer (II) containing KOH in the inner cup for the measurement of respiration (x_{O_2}) during the experimental period. Assuming R.Q. = 1, the respiratory $\text{CO}_2 = -x_{\text{O}_2}$. If the observed pressure changes and vessel constants are denoted by dashes for their respective vessels, and the aerobic fermentation by x_{FCO_2} , then

$$x_{\text{FCO}_2} = k'_{\text{CO}_2} \left(h' - \frac{h'' k'_{\text{CO}_2}}{k'_{\text{O}_2}} \right) + h'' k''_{\text{O}_2}.$$

Not every type of yeast responds in the manner described when thiamine is added, and yeast which does respond to the addition of thiamine may differ widely with regard to the time at which the increase of fermentation occurs. Some samples of baker's yeast have been tested at which the characteristic curves have been obtained only after 6-7 hr. incubation with thiamine. In such cases the increase of fermentation by thiamine (0.01-0.04 $\mu\text{g.}$) during the first hours of the experiments was either absent or more or less uniform at between 10 and

20 %, thus not permitting any distinction between the various amounts of thiamine. After 6–7 hr. the different rates became more and more distinct. In some cases incubation was extended for 24 hr., by which time the differences were very marked. The maximum increase of fermentation caused by the addition of 0.04 $\mu\text{g.}$ seldom exceeded 120–140 % however.

Behaviour of Torula utilis. *Torula utilis* did not respond at all within the period examined (24 hr.) to the applied range of thiamine (0.01–0.04 $\mu\text{g.}$). Neither aerobic nor anaerobic fermentation is increased above that of the control. On the other hand the rate of anaerobic fermentation of the control without thiamine increases steadily with time by approximately 20–25 % per hr. until it reaches a maximum of approximately 600 % at which level it seems to remain steady. *T. utilis*, without the addition of thiamine, behaves with regard

Fig. 6. Percentage increase of anaerobic fermentation caused by the same amount of thiamine (0.04 $\mu\text{g.}$) acting on different amounts of yeast. 20°.

to its anaerobic fermentation like baker's yeast to which thiamine has been added. This fact seems to justify the conclusion that *T. utilis* is able to synthesize thiamine from the medium and to do this at such a rate that added thiamine (0.01–0.04 $\mu\text{g.}$) does not further increase the already naturally increasing rate of fermentation. When 0.4 $\mu\text{g.}$ of thiamine was added an increase of fermentation of between 20–30 % was found only after more than 24 hr. incubation. Quantitative tests at this stage of incubation and with these larger doses have, however, not been made.

Torula has only a very small, practically insignificant, aerobic fermentation. Unlike the anaerobic fermentation, this does not increase with time. On the other hand respiration increases at about the same rate as anaerobic fermentation.

While the rate of anaerobic fermentation of *Torula* without thiamine increases, that of baker's yeast as a rule decreases slightly with time. In analogy

to the behaviour of *Torula* which probably synthesizes thiamine it might be concluded that baker's yeast is not only incapable of doing this but is losing some of its own preformed thiamine under the conditions of the experiment.

If this is the case, then different amounts of yeast incubated for long periods with the same amount of thiamine should show differences in the increase of CO_2 production. In the experiment summarized in Fig. 6 a sample of baker's yeast was used which responded only very slowly and which reacted to different amounts of thiamine in the characteristic way only after prolonged incubation. Different amounts (1.5, 3 and 6 mg.) of this yeast were exposed to the same amount of thiamine ($0.04 \mu\text{g.}$) and the relative increase of CO_2 production determined at different times. Since the volume of fluid was the same for each vessel, the relative amounts of added thiamine per mg. of yeast were in the ratio of 1 : 2 : 4. If preformed thiamine was used up at the same rate in each vessel, then the relative increase with time of CO_2 production caused by $0.04 \mu\text{g.}$ should be greater with 1.5 mg. than with 6 mg. yeast. This is actually the case, as can be seen from Fig. 6.

The effect of reduced thiamine

Lipmann [1936], who studied the reduction of vitamin B_1 by $\text{Na}_2\text{S}_2\text{O}_4$ and by H_2 in presence of platinum black, remarked on the close similarity of his results to those of Warburg *et al.* [1935] with coenzyme. He suggested that the sulphite-reduced compound corresponds to the naturally reduced component of thiamine in the body and assumed that thiamine in the body is reduced and reoxidized. It was therefore of interest to compare the influence of the oxidized and reduced product on the rate of fermentation of yeast. The following results have been obtained.

(1) The reduction of thiamine by H_2 + platinum black yields an irreversibly inactive product, e.g. fermentation in presence of H_2 -reduced thiamine, even over prolonged periods, is of exactly the same magnitude as that of the control without thiamine.

(2) When thiamine which has previously been reduced by $\text{Na}_2\text{S}_2\text{O}_4$ (in 0.2 % NaHCO_3) is added to a readily reacting type of yeast in N_2 (by tipping it from a side bulb) no difference is found as compared with the effect of the oxidized compound. It must be assumed, therefore, that reduced thiamine is rapidly reoxidized within the yeast cells. The smallest amount of yeast used for these experiments was 1.5 mg. with $0.04 \mu\text{g.}$ reduced thiamine. If still smaller amounts of yeast could be used, a time lag for the complete reactivation of the reduced thiamine might be expected and a quantitative relation established. The technical difficulties, however, in determining this relation seemed unsurmountable: this is not surprising considering that the analogous reoxidation of reduced coenzyme by flavin comes to a standstill within 5 min.

It may be added that $\text{Na}_2\text{S}_2\text{O}_4$ is itself not without influence on the metabolism of yeast. Even in very small amounts it inhibits respiration and fermentation, the latter to a greater extent. No effect on either respiration or fermentation was obtained when 0.01 mg. or less $\text{Na}_2\text{S}_2\text{O}_4$ was added per 3 ml. of fluid. The reduction of thiamine was therefore carried out in a relatively concentrated solution of thiamine, e.g. 1 ml. 0.2 % NaHCO_3 contained $10 \mu\text{g.}$ thiamine and 2.5 mg. $\text{Na}_2\text{S}_2\text{O}_4$. This was diluted 50 times, and 0.2 ml. was then tipped from the side bulb of the vessel into the yeast suspension under anaerobic conditions. The same amount of equally diluted NaHCO_3 solution was tipped into the control vessels without thiamine and with oxidized thiamine.

CONCLUSIONS AND SUMMARY

1. Under the conditions used by Schultz *et al.* thiamine in amounts as low as 0.01 μg . increases the anaerobic fermentation of yeast.

2. The percentage increase of the rate of fermentation by 0.01–0.04 μg . thiamine, plotted against thiamine concentration for several consecutive periods of the experiment, gives a similar type of curve. Thus it is possible to make quantitative tests of unknown concentrations of thiamine within this range with considerable accuracy.

3. Not every type of yeast responds with increased fermentation to the addition of thiamine. Thus baker's yeast was found to react regularly and fairly well, while *Torula utilis* under similar conditions did not respond at all.

4. The time at which responding yeast begins to show an increased fermentation after thiamine has been added varies considerably. Some samples react immediately, some only after several hours of incubation.

5. Once the rise of fermentation has started it increases with time for the same dose of thiamine. Differences in the increase of fermentation caused by different amounts of thiamine acting on the same amount of yeast are therefore more pronounced (and easier to evaluate quantitatively) after some incubation, although the relative increases remain the same.

6. In yeasts which react by an increase of anaerobic fermentation, aerobic fermentation is also increased quantitatively by the same minute amounts of thiamine, the percentage increase being of about the same magnitude as that of anaerobic fermentation.

7. The reaction which causes the increase of fermentation has a low temperature coefficient.

8. Different amounts of a slowly reacting sample of yeast incubated with the same amount of thiamine for 24 hr. show, as time goes on, a relatively greater increase of fermentation with decreasing amounts of yeast.

9. Reduction of thiamine by H_2 in presence of platinum black yields an irreversibly inactive product.

10. The $\text{Na}_2\text{S}_2\text{O}_4$ -reduced thiamine, when added to yeast in N_2 , affects fermentation in the same way as the oxidized form. It is concluded that the reduction by $\text{Na}_2\text{S}_2\text{O}_4$ is reversible and that thiamine reduced by $\text{Na}_2\text{S}_2\text{O}_4$ is reoxidized within the living yeast cell.

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Note added 26 May 1941. The application of the yeast fermentation method for the quantitative assay of vitamin B_1 in foodstuffs and tissues is limited by the fact that some yeasts are capable of synthesizing vitamin B_1 from certain degradation or split products of the vitamin molecule. These products, however, are not biologically active in animals. The presence of some such products might therefore give a higher fermentation test than warranted by the presence of free thiamine. I am indebted to Dr L. Harris who kindly drew my attention to this point.