Studies on Yeast Metabolism

5. THE TREHALOSE CONTENT OF BAKER'S YEAST DURING ANAEROBIC FERMENTATION*

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In a previous study of the synthesis of cellular carbohydrate during fermentation by D.C.L. baker's yeast (Trevelyan, Gammon, Wiggins & Harrison, 1952) little variation was found in the trichloroacetic acid-soluble carbohydrate, of which the main constituent is trehalose (Trevelyan & Harrison, 1952). The low concentration of glucose in the medium used, about 5 mM, was thought to have restricted trehalose synthesis. The present paper reports measurements of trehalose in yeast during fermentation of high concentrations of sugar. As Brandt (1941) reported that trehalose disappeared rapidly from the yeast cell as a result of growth in a suitable medium, but increased in amount during metabolism in the absence of assimilable nitrogen, particular attention has been paid to the effect on yeast trehalose of nitrogenous constituents of the medium. Disappearance of trehalose during budding has been noted also by Pollock & Holmstrom (1951). Recently a study of some aspects of the connexion between carbohydrate and nitrogen assimilation in *Torulopsis utilis* has been published by Yemm & Folkes (1954).

METHODS

Fermentation was carried out at 30°, using, unless otherwise stated, a suspension of 2.5 g. (wet wt.) of pressed D.C.L. baker's yeast/80 ml. This was stirred by a stream of 150 ml. of N₂/min. Four separate batches of yeast (referred to below as yeast I, II, III or IV) were used in the investigation; one (yeast IV) had a much higher trehalose content than the rest. Fermentation was initiated by adding 20 ml. of sugar solution. After allowing 2 min. for mixing, the first sample was withdrawn for carbohydrate determination.

Media. The 'full medium' contained 0.05M sodium succinate buffer, pH 5.5, 2 mM-KH₂PO₄, 8 mM-KCl, 5 mM-MgSO₄, 0.01M-(NH₄)₂SO₄, 0.02M DL-asparagine and 5 μM thiamine. The 'N-free medium' had no ammonium salts or asparagine. The 'P-free medium' contained 0.01M-KCl, but no KH₂PO₄. The 'asparagine-free medium' had 0.05M-NH₄Cl as sole nitrogen source. The fermentable sugars used were glucose, fructose or mannose, in an initial concentration of 0.3M (unless stated otherwise) and sucrose or maltose, 0.15M. In the most rapid fermentation the

glucose concentration at the end of a 2 hr. experimental period was still enough to saturate the yeast (0.15M).

Determination of carbohydrate. Trevelyan & Harrison (1952) described a rapid method for the routine determination of yeast carbohydrates based on the application of the anthrone technique either directly to the whole cells or to extracts containing principally trehalose, glycogen, mannan or glucan. The principles of this method were followed in the present work. Colour was developed, however, by the heat-of-mixing technique of Morris (1948). To determine TCA (trichloroacetic acid)-soluble carbohydrate (trehalose), 2 ml. of yeast suspension were pipetted into 3 ml. of ice-cold water: the cells were separated by centrifuging and washed twice with 5 ml. of cold water. The washed cells were then extracted three times with 4 ml. 0.5M TCA, each extraction taking 20 min. The combined supernatants were diluted to 50 ml., and the carbohydrate content was measured by the anthrone method.

TCA-insoluble carbohydrate, which includes the polysaccharides glycogen, mannan and glucan (Trevelyan & Harrison, 1952), was determined by applying the anthrone technique directly to a suspension in water of the TCA-extracted cells. As it was thought that some loss of cellular carbohydrate might occur during washing of yeast with water before TCA extraction, a few determinations of TCA-insoluble carbohydrate were made in which the yeast sample was delivered directly into TCA to destroy instantaneously all enzyme action. Results were essentially unaltered, so this procedure was abandoned, as trehalose could not be determined on the same yeast sample.

Results are given as μmoles glucose/g. original wt. of yeast.

Fermentation rate. This was determined, in separate experiments, by absorption of CO₂ in alkali and titration of the carbonate after precipitation with barium ions (Trevelyan & Harrison, 1954a).

RESULTS

Identification of the TCA-soluble carbohydrate as trehalose

Resting yeast had been previously examined (Trevelyan & Harrison, 1952), but it seemed possible that fermentation, particularly of sugars other than glucose, might lead to an alteration in the nature as well as amount of the TCA-soluble carbohydrate. To test this, yeast after fermentation for 2 hr. in an N-free medium, of glucose, fructose, mannose or

* Part 4: Trevelyan & Harrison (1954b).

maltose, was washed and then extracted with 1 ml. of M TCA/g. yeast. Carbohydrates in the extract, before and after hydrolysis for 2 hr. at 100° in N -HCl, were identified by paper chromatography (Partridge, 1948). After development in a pyridine-butanol-water (20:30:13, v/v) solvent each extract gave a spot with an R_f value characteristic of trehalose, which reacted weakly with the silver nitrate reagent of Trevelyan, Procter & Harrison (1950). After hydrolysis this was replaced by a rapidly reacting spot with the R_f value characteristic of glucose. Glucose was identified in the hydrolysates also after development of chromatograms in a phenol-isopropanol-water (70:5:25, v/v) solvent, which gave a better separation of glucose and fructose. All chromatograms showed faint spots with R_f values smaller than that of trehalose, and the number and intensity of these were much increased in an extract of a yeast which had fermented glucose in a medium containing assimilable nitrogen. As these spots reacted with ninhydrin they were probably due to amino acids. However, as a further check, a heavily loaded chromatogram was cut into strips, and material on the paper was dissolved in water and tested with the anthrone reagent. Only from the trehalose region was anthrone-positive material obtained.

Trehalose breakdown and resynthesis during fermentation of glucose

In a preliminary experiment a high concentration of yeast (10%, w/v) was used, so that extracellular glucose was exhausted after 80 min. The medium contained glucose (0.2M), but none of the salts or other constituents listed under Methods. Fermentation, for the first 40 min., was accompanied by a partial breakdown of yeast trehalose (Fig. 1). The subsequent resynthesis was interrupted by the exhaustion of fermentable substrate at 80 min., after which the level of trehalose was fairly stable. (Spiegelman & Nozawa (1945) have reported that yeast will not metabolize its intracellular carbohydrate in the absence of fermentable substrate.) During the initial period of trehalose breakdown the fermentation rate of the yeast increased (Fig. 1), and minimum content of trehalose coincided with the attainment of maximum fermentation rate.

Inhibition of trehalose resynthesis during assimilation of nitrogen by yeast fermenting glucose

That trehalose metabolism is not, however, linked in a simple manner with the fermentation rate was shown by comparing trehalose measurements made on yeast fermenting glucose in the different media described under Methods. These supported quite different fermentation rates, though in each case an initial period of rapidly

rising fermentation rate could be distinguished. After this initial phase the rate remained constant at $55 \mu\text{moles CO}_2/\text{min./g.}$ yeast in thiamine-free medium. After fermentation for 35 min. in N -free medium the rate had reached its maximum ($69 \mu\text{moles of CO}_2/\text{min./g.}$), after which time it declined by about 10% per hour. In P -free medium (at 20 min.) the rate was $61 \mu\text{moles of CO}_2/\text{min./g.}$ The rate continued to increase; the acceleration, however, rapidly died away to give

Fig. 1. Trehalose breakdown and resynthesis during fermentation of glucose by 10% (w/v) yeast II. Medium, 0.2M glucose, no other additions. O, yeast trehalose; ●, fermentation rate; ×, extracellular glucose.

at a rate of only $74 \mu\text{moles of CO}_2/\text{min./g.}$ after 120 min. of fermentation. Enzyme synthesis occurring in full medium supported a linear increase of rate from $77 \mu\text{moles of CO}_2/\text{min./g.}$ at 20 min. to 114 at 120 min. As may be seen from Figs. 2 and 3, yeast trehalose broke down in the initial stage of fermentation, and to a greater extent than when a simple solution of glucose had been used as the fermentation medium (cf. Fig. 1). Appreciable resynthesis was observed only when assimilable nitrogen was omitted from the medium. In thiamine-free medium the fermentation rate was lower than in N -free medium, but only a slow resynthesis of trehalose was observed; in full medium the rate was much higher, but again resynthesis was inhibited. The substitution of casein hydrolysate for asparagine and ammonium sulphate was without effect (Fig. 3). By comparing the composition of yeast after fermentation of glucose for 2 hr. in (a) full medium and (b) N -free medium, it was found that the incorporation (mainly as protein) into the acid-insoluble fraction of $1 \mu\text{mole}$ nitrogen

reduced the amount of glucose assimilated as trehalose and polysaccharides by about $0.5 \mu\text{mole}$. This corresponds to the replacement of carbohydrate formation by synthesis of an equal weight of protein.

Fig. 2. Effect of constituents of the medium on trehalose breakdown and resynthesis during fermentation by yeast I. $\circ$, Full medium + glucose (0.3M); $\times$, P-free medium + glucose (0.3M); $\square$, N-free medium + glucose (0.3M); $\bullet$ (upper curve), N-free medium + maltose (0.15M); $\circ$ (lower curve), full medium + maltose (0.15M).

Fig. 3. Effect of constituents of the medium on trehalose breakdown and resynthesis during fermentation of glucose (0.3M) by yeast III (cf. Fig. 2). $\circ$, Full medium; $\bullet$, N-free medium + amino acids (casein hydrolysate, 0.05M in terms of total N, $+0.5\text{ mM}$ L-tryptophan); $\times$, thiamine-free medium.

Stability of trehalose in the absence of fermentable substrate

Yeast trehalose was stable when incubated with ammonium sulphate (asparagine-free medium) in the absence of glucose. Seven determinations of trehalose content, made at 20 min. intervals, gave the mean value of $143 \mu\text{moles}$ (as glucose)/g., with an S.D. of ± 5 . This experiment provided incidentally a fair test of the reproducibility of the analytical procedure.

Relation between the initial breakdown of trehalose and the metabolism of intracellular nitrogen reserves

It is natural to inquire whether nitrogenous constituents of the yeast itself are responsible for the initial breakdown of trehalose, which occurred

Fig. 4. Elimination of the initial phase of trehalose breakdown during fermentation in N-free medium (0.3M glucose), by pre-treating yeast IV for 60 min. with glucose in full medium ($\circ$), or for 80 min. with glucose in N-free medium ($\bullet$). After pre-treatment the yeast was washed and transferred to fresh N-free medium.

even in N-free medium. As washing the yeast with 40 times its bulk of N-free medium before fermentation did not eliminate trehalose breakdown (Fig. 6), contamination of the yeast by extracellular nitrogen compounds was ruled out. Yeast, however, contains intracellular amino acids; the composition and metabolism of this nitrogen reserve have been considered by Halvorson & Spiegelman (1953). Partial depletion of the amino acid reserve was brought about by fermentation in N-free medium, as shown by a decrease in the intensity of spots on a paper chromatogram of TCA extracts prepared as described under Methods and sprayed with ninhydrin. After fermentation for 80 min., when the

trehalose level of the yeast had returned to its initial value, the yeast was separated by centrifuging and washed free of medium. A second sample of yeast, fermented for 60 min. in full medium, retained its nitrogen reserves. When glucose in N-free medium was added to both samples of yeast, the trehalose level immediately began to increase (Fig. 4). The difference between the rates of synthesis in the two cases was not sufficient to support the idea of a relation between trehalose breakdown during fermentation by untreated yeast and the metabolism of intracellular amino acid reserves.

Fig. 5. Interruption of trehalose resynthesis (yeast IV) by addition of $0.05\text{M-NH}_4\text{Cl}$ ($\downarrow$) after fermentation of 0.3M glucose had proceeded for 80 min. in N-free medium (O): for comparison, the progress of trehalose resynthesis during fermentation in N-free medium is plotted (dotted line). ●, Failure of mM uridine or adenine (identical results) to promote trehalose resynthesis during fermentation of 0.3M glucose in full medium.

It will be recalled that the initial period of trehalose breakdown was also one of increasing fermentation rate. Treatment of the yeast with glucose in the manner described abolished not only trehalose breakdown, but also the lag in attaining the maximum rate of fermentation (Trevelyan & Harrison, 1954a).

Further experiments on the effects of nitrogen assimilation on trehalose metabolism

After fermentation of glucose for 80 min. in an N-free medium, the yeast had resynthesized approximately the amount of trehalose lost in the initial breakdown period. Ammonium chloride added at this point immediately caused the level of yeast trehalose to decline once more (Fig. 5).

It was thought possible that competition between the reactions of trehalose and protein synthesis for common coenzymes, such as the adenine nucleotides or uridine diphosphoglucose [reported by Leloir & Cabib (1953) to be involved in the synthesis of trehalose], might explain the antagonistic effects of nitrogen assimilation on trehalose synthesis. Uridine or adenine, possible precursors of coenzymes, were therefore added to yeast fermenting glucose in full medium, but no effect was observed (Fig. 5). In this experiment only, trehalose was determined immediately before the addition of glucose as well as 2 min. afterwards. Even in 2 min. the breakdown of yeast trehalose is appreciable (Fig. 5).

Trehalose levels during fermentation of maltose

Maltose was fermented much more slowly than glucose, and the rate increased with time owing to synthesis of the adaptive enzyme maltase. Only minor changes in yeast trehalose accompanied maltose fermentation (Fig. 2), though a slow breakdown of trehalose in the second hour was induced by addition to the medium of assimilable nitrogen.

Trehalose levels during fermentation in N-free medium of sucrose, fructose, or mannose

Glucose could be replaced by sucrose, fructose, or mannose without significant alteration in fermentation rates. In each case (Fig. 6) an initial period of trehalose breakdown was succeeded by resynthesis. With fructose or mannose, differences were recorded between the minimum trehalose level and the rates of resynthesis, as compared with those characteristic of glucose fermentation.

Effect of azide

It was shown previously (Trevelyan *et al.* 1952) that above a certain concentration sodium azide induced a breakdown in yeast trehalose during fermentation of glucose. As Fales (1953) obtained different results, possibly owing to his using a different type of yeast, the effect of azide on trehalose metabolism was re-investigated. Previous results were confirmed: as Fig. 7 shows, mM- NaN_3 caused the breakdown of trehalose and of part of the TCA-insoluble carbohydrate (polysaccharides) when added to yeast fermenting glucose in N-free

medium either 2 min. before sugar addition, or after fermentation had proceeded for 60 min. (This concentration of azide inhibited fermentation somewhat, the respective rates being 40 and 50 μ moles of CO₂/min./g. as compared with the control rate of over 60.) Azide action on trehalose metabolism is particularly interesting, since this

inhibitor has been shown to affect enzyme (protein) synthesis by yeast (Spiegelman & Reiner, 1947).

Level of TCA-insoluble carbohydrate

The changes of yeast trehalose level during the fermentation of glucose were accompanied by similar, though less striking, variations in TCA-insoluble carbohydrates (Table 1). Later experiments (unpublished) have shown these changes to be due largely to alterations in glycogen content.

DISCUSSION

The enzyme systems involved in the synthesis and degradation in the yeast cell of trehalose (or indeed of any other yeast carbohydrate) have not been worked out in detail. The overall kinetics, e.g. of

Fig. 6. Trehalose content of yeast IV during fermentation, in N-free medium, of O, fructose; ●, glucose; ×, mannose; □, sucrose. Before the glucose fermentation, the yeast was washed to ensure that possible extra-cellular contaminants had no influence on the initial trehalose breakdown.

Fig. 7. Breakdown in yeast IV of trehalose (O) and TCA-insoluble carbohydrate (including glycogen) (●), in the presence of mm sodium azide. Full line: azide added 2 min. before glucose. Dotted line: azide added 60 min. after glucose (↑).

Table 1. *Synthesis of yeast TCA-insoluble carbohydrate during fermentation of glucose (G), maltose (MA) or mannose (M)*

(a) marks estimations in which yeast was delivered directly into trichloroacetic acid (see Methods).

Yeast (no.)	...	I	I	I	I	III	III	III	IV
Sugar fermented	...	G	G	G	MA	G	G	G	M
Medium	...	Full	P-free	N-free	N-free	Full	Thiamine-free	Full, casein hydrolysate as N source	N-free
TCA-insoluble carbohydrate [μ mole (as glucose)/g. yeast]									
Time (min.)	(a)	(a)							
2	344	341	310	344	309	320	309	378	
20	325	341	335	346	282	311	286	393	
40	348	371	416	358	309	338	284	413	
60	355	390	494	373	317	359	308	480	
80	363	409	529	401	338	371	348	528	
100	393	431	580	431	352	389	361	585	
120	428	468	593	455	398	416	385	645	

trehalose metabolism, involve not only the kinetics and equilibrium conditions of these systems, largely unknown, but also the concentrations of intermediates such as glucose 6-phosphate which are maintained at a steady level in the cell as a result of the complex interaction of several or all of the enzyme systems of the Embden-Meyerhof-Parnas scheme.

The formation of trehalose 6-phosphate during fermentation by dried yeast or cell-free yeast extracts (Myrbäck, 1949) suggests that this substance plays an important role. Recently (P. F. E. Mann & J. S. Harrison, 1955, private communication) an improved version of the ion-exchange technique used by Trevelyan, Mann & Harrison (1954) has been applied to show that trehalose 6-phosphate is formed in the yeast cell within 10 min. of adding glucose. A simple explanation of the stability of trehalose in the absence of fermentable substrate would be to postulate an initial phosphorylation to trehalose 6-phosphate as the initial step in the breakdown of this carbohydrate. This type of explanation is difficult to apply to the case of glycogen, which, as we have seen, behaves similarly to trehalose during fermentation.

Nitrogen assimilation involves the formation of amino acids, principally from intermediates formed by reactions branching off the main pathway of fermentation at the pyruvate level. The reactions of glycogen, and probably trehalose synthesis (Myrbäck, 1949), diverge at the glucose 6-phosphate level. It is at first sight difficult to see how these two groups of synthetic reactions can interact. That an indirect effect of nitrogen assimilation on carbohydrate formation is, in principle, possible is shown by considering a property of the ammonium ion not directly related to its function as a source of assimilable nitrogen. Muntz (1947) reported that NH_4^+ (and K^+) stimulated phosphofructokinase in yeast-maceration juice. The effect of such stimulation, occurring during fermentation *in vivo*, would be either to increase the rate of fermentation or, if the rate is restricted by reactions other than the phosphofructokinase system, to lower the steady-state level of fructose 6-phosphate and indirectly of glucose 6-phosphate. Conditions would then be less favourable to glycogen or trehalose synthesis.

Phosphofructokinase is linked to hexokinase through a common coenzyme, adenosine triphosphate (ATP). Theoretically, the steady-state level of glucose 6-phosphate during fermentation should be regulated not so much by the absolute amount of phosphofructokinase, as by its amount relative to hexokinase. This latter enzyme has different reaction rates and Michaelis constants towards glucose, fructose and mannose (Slein, Cori & Cori, 1950), conceivably accounting for the differences in

trehalose levels found during fermentation of the different sugars.

Since a period of adaptation to the medium eliminates the initial phase of trehalose breakdown when glucose is added to the yeast in a nitrogen-free medium, it is suggested that ionic exchanges between the yeast and the medium are involved in the transition from conditions favouring trehalose breakdown to conditions favouring synthesis during the first 30 min. of glucose fermentation. Alterations in the internal concentration of ions such as K^+ , Mg^{2+} , H_2PO_4^- may, through effects on hexokinase and phosphofructokinase, and possibly other reactions of alcoholic fermentation, alter the steady-state levels of glucose 6-phosphate and those of other intermediates important in carbohydrate assimilation.

Since both carbohydrate and nitrogen assimilation are processes which utilize the adenosine-triphosphate (ATP) generated during fermentation, the inhibition of carbohydrate reserve formation during (anaerobic) nitrogen assimilation may be regarded as a mechanism ensuring the distribution of the limited amount of ATP available for synthetic reactions in the most biologically effective manner.

SUMMARY

1. Fermentation of glucose, sucrose, fructose or mannose by baker's yeast is accompanied by an initial breakdown in yeast trehalose.

2. In nitrogen-free medium this initial breakdown is succeeded by trehalose resynthesis. Assimilable nitrogen prevents resynthesis during fermentation of glucose for 2 hr.

3. After fermenting glucose with or without assimilable nitrogen yeast transferred to fresh nitrogen-free medium starts synthesizing trehalose immediately glucose is added.

4. Yeast trehalose shows only minor changes during fermentation of maltose.

5. The breakdown of yeast trehalose during glucose fermentation in presence of mm azide has been confirmed.

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Studies on Yeast Metabolism

6. EFFECTS OF THIAMINE ON NITROGEN ASSIMILATION DURING FERMENTATION, AND ON FERMENTATION KINETICS

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Trevelyan & Harrison (1954*a, b*) showed that the addition of thiamine brought about a rapid lowering in the steady-state concentration of pyruvate in the yeast cell during fermentation, accompanied by only a slight immediate increase in fermentation rate. They suggested that carboxylase could therefore be considered to be one of the factors limiting fermentation rate only when thiamine was omitted from the medium, and that some other enzyme system or systems limited the fermentation rate of thiamine-saturated yeast.

Thiamine, in an appropriate medium, is known to promote an increase in fermentation rate which continues for several hours. This long-term action of thiamine, which was discovered by Atkin, Schultz & Frey (see their review in Anderson, 1946), is considered in the present paper in relation to the short-term effect on pyruvate levels mentioned above.

While this work was in progress, a paper by Maesen (1953) appeared in which it was reported that the long-term increase in fermentation rate mediated by thiamine was accompanied by a proportionate increase in the carboxylase activity of the yeast cells (determined after disruption by freezing and thawing). Rate increases were observed only when assimilable nitrogen (ammonium sulphate) was supplied to the yeast, but the rate of its incorporation into yeast protein was unaffected by thiamine. Thiamine thus appeared to have a specific effect on carboxylase synthesis, and this enzyme system was, Maesen concluded, the limiting factor of fermentation under his experimental conditions.

METHODS

Fermentation technique

Yeast was taken as required from a master sample of D.C.L. pressed baker's yeast stored in the refrigerator over a period of not more than 3 weeks. When necessary, the various master samples are distinguished by the letters A, B etc.

Full medium M1 contained sodium succinate buffer, pH 5.5 (0.05 M succinic acid partly neutralized with 75 mM-NaOH), 0.01 M-KH₂PO₄, 0.01 M-(NH₄)₂SO₄, 0.02 M DL-asparagine, 2.5 mM-MgSO₄, mM-ZnSO₄, mM-CaCl₂ and 0.1 μmole thiamine/g. yeast. Medium M2 contained sodium succinate buffer, ammonium sulphate and asparagine in identical concentration: the other constituents were 5 mM-MgSO₄, 2 mM-KH₂PO₄, 8 mM-KCl and 0.2 μmole thiamine/g. yeast. Essentially similar rates were obtained during fermentation for 2 hr. with the two media. Thiamine-free medium, N-free medium, P-free medium contained all constituents except thiamine, ammonium sulphate and asparagine, or phosphate, respectively. Asparagine-free medium had 25 mM-(NH₄)₂SO₄ as sole nitrogen source.

Fermentation was carried out at 30°, the suspension being stirred by bubbling N₂ through at about 150 ml./min. Variation of the gas flow between 100 and 200 ml./min. had no effect on the fermentation rate. Unless stated otherwise, the fermentable sugar was 0.3 M glucose. Usually the final volume of 100 ml. contained 2.5 g. (fresh wt.) of yeast, which had a dry-matter content of 27%. In previous studies of the short-term effect of thiamine, double this concentration of yeast was used (Trevelyan & Harrison, 1954*b*). Although the fermentation rate/g. of yeast was the same during the first 40 min. of fermentation, if 2.5 g. or 5 g. of yeast were used, it was found that a steady increase in rate after this time was observed only at the lower level of yeast concentration.

Fermentation rate, as an average over 10 or (normally) 20 min. intervals, was determined after absorption of CO₂ in alkali (Trevelyan & Harrison, 1954*a*).