The Uptake and Incorporation of ¹⁴C-labelled Sugars into Polysaccharides during Wort Fermentation by a Synchronous Culture of Brewer's Yeast

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SUMMARY

The incorporation of [U-¹⁴C]glucose, -maltose and -maltotriose into the polysaccharides of a brewer's yeast, *Saccharomyces cerevisiae* (NCYC240), in synchronous culture was followed during the fermentation of brewer's wort. The synchrony was induced by repeated subculture in brewer's wort at regular intervals, using cells skimmed from the surface of previous brews. The ¹⁴C-labelled sugars were assimilated simultaneously by the yeast and small but significant amounts of each incorporated into cellular polysaccharides. The low molecular weight acid-soluble fraction exhibited a cyclic fluctuation attributable to changes in enzymic activity associated with the synchronous behaviour of the yeast. Cyclic changes were also observed in the incorporation of labelled sugars into the mannan fraction of the walls, the uptake rising to successive maxima at the same stage of each budding cycle, followed by a rapid loss as the next cycle began. The incorporation of ¹⁴C into glycogen exhibited a stepwise character, in which brief periods of glycogen build-up alternated with longer periods of stability. The periods of glycogen biosynthesis coincided with the periods of most intensive budding. Incorporation of ¹⁴C into the glucan of the walls was a continuous process and could not be correlated with any particular phase of the cell cycle. Incorporation of ¹⁴C into glycogen and glucan was stable.

INTRODUCTION

Laboratory fermentation results have suggested that brewing yeasts metabolize the three principal sugars of brewer's wort in a sequential fashion (Harris, Barton-Wright & Curtis, 1951; Phillips, 1955; Harris & Millin, 1963; Millin, 1963, 1966; Walkey & Kirsop, 1969; Kirsop, 1971). Most results suggest that glucose is metabolized first, inhibiting the yeast from utilizing both maltose and maltotriose. After the glucose has disappeared maltose is utilized, but this inhibits the uptake of the maltotriose which is metabolized last of all (Kirsop, 1971). This hypothesis has since been modified, in particular by the suggestion that maltose and maltotriose might inhibit each other's uptake (Visuri & Kirsop, 1970) or that both are sometimes utilized simultaneously (Kirsop & Brown, 1972). The possible causes of the sequential sugar uptake are obscure; little or no experimental data are available, except for that of Portno (1968) who described diauxic behaviour in yeasts cultured in synthetic media and Griffin (1970*b*) who showed that a concentration of glucose in synthetic media above 0.3 to 0.4 % blocked the uptake of maltose by yeast. Other work, in which the sugar profiles of brewer's worts have been followed during the course of both laboratory (Budd, 1974) and brewery (Patel & Ingledew, 1973) fermentations, suggests that the three principal sugars of brewer's wort might be utilized simultaneously.

Adding labelled sugars to wort before fermentation, and then following the rate at which

label is incorporated into yeast polysaccharides, helps to resolve the question of whether brewery yeasts utilize the principal wort sugars simultaneously or sequentially, assuming that the rate of incorporation is proportional to the rate of utilization. There are differences of opinion as to the value of following the uptake of labelled sugars into yeast cell polysaccharides (Sentandreu & Northcote, 1969; Visuri & Kirsop, 1970) but previous work in which the incorporation of ^{14}C -labelled glucose into yeast polysaccharides was followed suggested that some useful results could be obtained. The incorporation of labelled material into mannan and a low molecular weight fraction was impermanent, whereas label once incorporated into glycogen and glucan was permanent (Budd, 1974). The turnover in the low molecular weight fraction was due to its transient biochemical status, whereas the mannan turnover was attributed to the shedding of phospho-mannan-protein complexes from the wall into the medium. These results suggested that additional information might be obtained by using ^{14}C -labelled maltose and maltotriose as well, and that more meaningful data might be obtained if the yeast could be induced to behave in a synchronous manner during the wort fermentation.

Synchrony in yeast has recently attracted much interest (see reviews by Duffus, 1971; Hartwell, 1974). The pattern of events during each cell cycle is highly temperature-dependent (Mitchison, Kinghorn & Hawkins, 1963), and most studies have unfortunately been done at temperatures of 30 °C or above, which makes their direct application to brewing conditions questionable. Most ale fermentations are conducted at 17 to 23 °C, and most lager ones at 8 to 10 °C. Little or no published work on yeast synchrony has been carried out using brewer's yeast or under brewing conditions, and no work involving the polysaccharides of synchronous yeast cultures has been reported. The work reported here seeks to rectify some of these omissions.

METHODS

Induction of synchrony. Brewer's yeast, *Saccharomyces cerevisiae* (NCYC240) was inoculated into brewer's wort (2.0 l; sp.gr. 1.040) in a tubular fermenter (Dixon, 1967) and allowed to grow and ferment undisturbed for seven days. The yeast, suspended in the stable surface foam (the head), was then skimmed off with a sterile spoon and immediately re-inoculated (pitched) into a fermenter containing fresh wort (2.0 l). This procedure was repeated many times, and resembles the skim and pitch routine practised in traditional ale brewing. Microscopic examination of the fermenting liquor showed that after about 10 or 12 subcultures, despite its chain forming habit (Brown, 1970) the yeast would sometimes exhibit a remarkable degree of synchrony, shown by the simultaneous appearance of new buds in many of the cells on re-pitching. A large proportion of the cells in the stable surface foam were without buds, although heterogeneous in size. The rapid onset of budding on re-pitching suggests that they were probably arrested in the interphase (Hartwell, 1974). Arrested cells were washed with 0.067 M- KH_2PO_4 at 5 °C and stored as a moist paste at 5 °C.

The synchronous fermentation. Brewer's wort (250 ml; sp.gr. 1.040) was inoculated with the synchronous yeast culture (0.6 g wet wt) and mixed thoroughly. Individual [^{14}C]sugar solutions (1.0 ml, 10 μCi) obtained from the Radiochemical Centre, Amersham, Buckinghamshire, were added to three 60 ml portions of this wort. Each of these cultures was then divided into twelve further portions (5.0 ml) and transferred to sterile 10.0 ml conical centrifuge tubes. Each tube was stoppered with cotton wool and incubated in a water bath at 18 °C. A fourth culture (60 ml) was incubated at 18 °C in a 100 ml measuring cylinder and the specific gravity and sugar concentrations measured daily.

Yeasts were harvested at timed intervals from the cultures containing radioactive sugars

Fig. 1

Fig. 2

Fig. 1. Utilization of the principal sugars of brewer's wort during primary fermentation. ○, Glucose; ●, maltose; ▲, maltotriose.

Fig. 2. Incorporation of [^{14}C]sugars into whole cells during the fermentation of brewer's wort by a synchronous yeast culture. ○, Glucose; ●, maltose; ▲, maltotriose. The arrows indicate the periods of most intensive yeast budding.

by centrifugation, washed once with KH_2PO_4 solution (3.0 ml, 0.067 M, 5 °C) and then resuspended in the same KH_2PO_4 solution (5.0 ml). A small sample (1.0 ml) of this washed suspension was centrifuged, the cells resuspended in water (0.25 ml) and the radioactivity measured by liquid scintillation. The remainder of the suspension was centrifuged and the cells killed by resuspension in trichloroacetic acid (5 %).

Yeast synchrony was monitored by frequent microscopic examination of samples taken from the fourth culture throughout the fermentation. The periods of intensive budding became less well-defined as the fermentation progressed. After three periods the cycle lost momentum, the yeast became senescent and passed into G1 arrest (Hartwell, 1974) and the surface foam became stabilized.

The fractionation and counting procedure. The yeast polysaccharides were extracted by a procedure based on the method of Trevelyan & Harrison (1956) and modified for use with ^{14}C -labelled sugars. The extraction and counting techniques are as described by Budd (1974).

Other analyses. Sugar levels in the fermenting liquor were measured in duplicate by g.l.c. (Clapperton & Holliday, 1968).

RESULTS

The utilization of wort sugars

After a brief initial lag the sugars began to be utilized, glucose disappearing quickly (within 20 h), whilst the major component, maltose, was exhausted at 70 h (Fig. 1). Maltotriose levels fell continuously, but the rate of utilization slowed down after 70 h and a proportion was still unused at 100 h. It is rare nowadays for commercial ale fermentations to exceed four days, and they may even be stopped after only two or three. The cessation of fermentative activity is usually achieved by cooling followed by separation of the yeast from the liquor either by skimming or centrifuging, thus postponing the uptake of any unused malto-

triose. The utilization of this sugar is regarded as a variable parameter, some strains of brewing yeast utilizing it completely in the initial fermentation whereas others utilize only a fraction of it (Harris & Thompson, 1960; Portno, 1968; Walkey & Kirsop, 1969). The surviving maltotriose subsequently disappears during the post-fermentation maturation period due to the activity of the cells left in suspension, or else survives the brewing process and goes to flavour the final product, particularly where 'keg' beer is concerned. Had the fermentation been allowed to continue it is possible that all of the maltotriose would eventually have disappeared. In brewery fermentations the first 25 h is the period of most rapid yeast growth (Brown & Kirsop, 1972) although growth usually continues for 50 h or more. The size of the yeast inoculum used in this work (the pitching rate) corresponded to normal brewery practice, so that during the fermentation the yeast would be expected to undergo three or four successive cycles of reproductive budding. If the rate of sugar uptake were proportional to the surface area of active yeast, the initial rate of uptake would be comparatively slow.

The profile (Fig. 1) does not support the theory of sequential utilization of wort sugars. It suggests that brewer's yeast is capable of utilizing all three principal sugars of wort at the same time. Similar profiles have been observed in a commercial lager brew (Patel & Ingle-dew, 1973) and in laboratory ale fermentations using fast fermenting strains of brewing yeast (Griffin, 1970a).

The incorporation of [^{14}C]sugars into whole yeast

There was an immediate, rapid and substantial uptake of all three labelled sugars into the yeast, confirming that they were used simultaneously (Fig. 2). All the results in this and subsequent Figures are expressed as percentages of the original wort count, and give a direct indication of the percentage of each wort sugar incorporated into the whole organisms or into each polysaccharide fraction. The uptake was stepwise, with short periods of very rapid assimilation alternating with longer periods of little or no uptake. There were three such 'steps', occurring at approximately equal intervals concurrent with the periods of most intensive budding, and which were due mainly to incorporation into the glycogen fraction (see Fig. 5).

The incorporation of [^{14}C]sugars into low molecular weight material

There was an immediate and extremely rapid uptake of all three labelled sugars into this fraction (Fig. 3a), emphasizing perhaps its transient metabolic status as the fraction through which all absorbed nutrients must pass. With all three sugars the uptake attained its maximum within a very short period. After the initial assimilation phase, the radioactivity in this fraction exhibited a cyclic fluctuation which continued for most of the fermentation period, the fluxions being closely similar for all three sugars. This was not observed in previous work (Budd, 1974), probably because the wort fermentation was then non-synchronous. Griffin observed fluctuations in the levels of certain yeast enzymes associated with the uptake and metabolism of maltose, which were quite rapid in synthetic media (Griffin, 1969) but much slower in brewer's wort (Griffin, 1970a). If the fluctuations shown in Fig. 3 were partly due to fluctuations in the levels of maltose-metabolizing enzymes, then the enzyme activity associated with the uptake and metabolism of glucose and maltotriose presumably should also oscillate in the same way. The low molecular weight fraction of the yeast carbohydrate consists mainly of trehalose (Trevelyan & Harrison, 1956) but it must also include all the transient metabolites of glycolysis together with other low molecular weight sugars in transit. Yeasts exhibit small fluctuations in metabolite concentrations during which glucose

Fig. 3. Incorporation of [^{14}C]sugars into (a) the low molecular weight fraction of the cells and (b) the mannan fraction of the cell walls, during the fermentation of brewer's wort by a synchronous yeast culture. ○, Glucose; ●, maltose; ▲, maltotriose. The arrows indicate the periods of most intensive yeast budding.

is consumed in a pulsatory manner (Betz, 1966). Certain enzymes of the glycolytic system may alternately be activated and inhibited by regular changes in the levels of ATP, ADP and AMP, giving rise to a 'metabolic flux', which would provide a tenable alternative explanation of the results shown in Fig. 3. Similar metabolic oscillations have been observed in yeasts by Pye (1969).

The incorporation of [^{14}C]sugars into mannan

[^{14}C]glucose was incorporated into the mannan fraction of the cell walls in a cyclic but less regular pattern (Fig. 3*b*). The incorporation maximum of the second cycle was approximately twice that of the first, presumably a consequence of the doubling of cell numbers. Incorporation of labelled material during the third cycle did not reach the level attained during the second. By this time the wort glucose had been exhausted, and the continued uptake of labelled material into this and the other polysaccharide fractions cannot easily be explained without invoking gluconeogenesis (Chapman & Bartley, 1969) probably with labelled ethanol as the starting material. Drews & Hessler (1967) suggested that ethanol is oxidatively assimilated by yeast to produce acetaldehyde, which is fed into the citric acid cycle via the glyoxylate shunt and then built up into polysaccharide.

[^{14}C]maltose, or substances derived from it, also participated in mannan biosynthesis during the third cell cycle. It is tempting to link the fluctuations described here with the metabolic fluxes described previously. The connexion could be direct, with an internal metabolite acting in an allosteric manner, or indirect, with both systems being controlled by some other factor. Labelled maltotriose did not participate in mannan biosynthesis.

Fig. 4. Incorporation of $[^{14}\text{C}]$ sugars into (a) the glycogen fraction of the cells and (b) the glucan fraction of the cell walls, during the fermentation of brewer's wort by a synchronous yeast culture. $\circ$, Glucose; $\bullet$, maltose; $\blacktriangle$, maltotriose. The arrows indicate the periods of most intensive yeast budding.

The incorporation of $[^{14}\text{C}]$ sugars into glycogen

There was substantial incorporation of label from all three sugars into glycogen in the first few hours, with no evidence of sequential uptake (Fig. 4a). Label from glucose and maltose was assimilated after the sugars had disappeared from the fermenting liquor (cf. Fig. 3b), lending further support to the hypothesis that gluconeogenesis was taking place. As in previous work (Budd, 1974; Patel & Ingledew, 1973) there was no evidence of any turnover or breakdown in the glycogen.

Clearly the stepwise accumulation of label in glycogen, quantitatively the most important polysaccharide fraction, was the chief component of the total uptake shown in Fig. 2. Each step concurred with each cell cycle, the rapid increases representing a limited period of glycogen synthesis. These periods coincided with those of most intensive budding and most rapid mannan turnover. It is surprising that glycogen biosynthesis is concentrated into so short a period, probably in the late interphase. DNA replication occurs in the same position in the sequence, at the initiation of budding in the G1 sector of late interphase (Duffus, 1971; Hartwell, 1974), suggesting perhaps that this is a period when polymer biosynthesis generally occurs. For the rest of the cycle it appears that glycogen biosynthesis is suppressed.

The incorporation of $[^{14}\text{C}]$ sugars into glucan

Uptake of all three labelled sugars into the wall glucan occurred simultaneously (Fig. 4b). Uptake of $[^{14}\text{C}]$ glucose was very rapid between 25 and 50 h but did not increase thereafter. Uptake of $[^{14}\text{C}]$ maltose was less rapid than glucose but continued for much longer and was still proceeding at 100 h. $[^{14}\text{C}]$ maltotriose uptake remained comparatively slow throughout the fermentation and ceased altogether at about 50 h. Label from glucose and maltose con-

tinued to be incorporated into the glucan long after they had disappeared from the wort, again suggesting that gluconeogenesis was occurring. There was no evidence for stepwise assimilation, no correlation with the synchronous behaviour of the cells and no sign of turnover, confirming previous observations (Budd, 1974).

DISCUSSION

It is tempting to link the cycles of incorporation observed with the budding cycles of laboratory yeast. But with one exception (Yamamoto & Inoue, 1966) other workers have used comparatively high fermentation temperatures (30 to 35 °C) and defined or semi-defined media (e.g. malt extract-yeast extract-glucose-peptone medium, MYGP; Wickerham, 1951) rather than brewer's wort. The yeast synchrony observed by Yamamoto & Inoue (1966) involved a bottom fermenting yeast, *Saccharomyces carlsbergensis*, in brewer's wort at 8 °C, i.e. a lager type of fermentation. These authors suggested that the synchrony was induced by the normal everyday operation of the brewery and this is supported by the present results.

One of the oldest and most reliable methods of inducing synchrony in laboratory yeast is that of Williamson & Scopes (1962), which involves transferring stationary-phase cells between growth and starvation media coupled with selection of the larger cells in the population. The nutritional deprivation produces stationary cells that are 'arrested' synchronously at a point in the cell cycle between cell separation and initiation of new DNA synthesis (the G₁ phase). It is possible that the formation of the stable surface foam in the tubular fermentations used in this work may have reproduced these conditions.

The more obvious implications of the results with yeast mannan (Fig. 3*b*) have already been mentioned. Total synchrony in a microbial population cannot be achieved, but had it been obtained in this work it is possible that the loss of labelling from the mannan might have been complete at the end of each cell cycle. Certain degradative enzymes, including mannanase, are produced by synchronously dividing yeast cultures, but only during the pre-budding phase of each cell cycle (Maddox & Hough, 1971). The activity of these enzymes results in the breaking of linkages in the wall polymers, allowing new units to be inserted and thus permitting the wall to be expanded to allow for growth (McClary & Bowers, 1965). The work of Maddox & Hough (1971), although carried out under non-brewing conditions in Wickerham's MYGP medium at 30 °C, nevertheless provides a good explanation of some of the results reported here. The most marked changes in labelling in both the mannan and glycogen fractions occurred when new buds were appearing most rapidly. Maddox & Hough (1971) suggested that the mannan, normally regarded as a wall polymer (Lampen, 1968), might be utilized during periods of carbohydrate deficiency since it disappeared completely from yeast during cold storage. Alternatively, the mannan might simply be discarded from the outer layers of the wall (Northcote & Horne, 1952; Lampen, 1968; MacWilliam, 1970; Hough, Briggs & Stevens, 1971).

Kuenzi & Fiechter (1969, 1972) investigated the carbohydrate composition of yeast cells during the cell cycle. In cells synchronized by glucose starvation and refeeding, the glucan and mannan contents remained relatively constant but the glycogen and trehalose contents decreased abruptly at the time of budding. The pattern of events seemed to depend on the methods employed to induce synchronization and these authors suggested that glycogen and trehalose might provide a source of energy for the budding processes under the special conditions of glucose limitation. The results reported here and previously (Budd, 1974) for brewer's yeast under brewery conditions, do not support the idea that glycogen is utilized

as an energy source, at least during the primary fermentation, but suggest that glycogen synthesis is a discontinuous process. The discontinuous or stepwise synthesis of enzymes is apparently quite general in *S. cerevisiae* (Halvorsen, Carter & Tauro, 1971) and is believed to come about as a result of the sequential transcription of chromosomes. The stepwise character apparent in the build-up of glycogen and mannan makes the enzymes involved obvious candidates for sequential transcription, but it is difficult to see how this idea could be invoked in glucan biosynthesis or sugar uptake, both of which appeared to be continuous processes. An alternative explanation is the controlled transcription of the genes concerned with glycogen and mannan biosynthesis. The production of many yeast enzymes is said to be controlled in this way (Gorman *et al.* 1964; Scopes & Williamson, 1964; Halvorsen *et al.* 1966). The alternative metabolic model of Rothman & Cabib (1967*a, b*), in which yeast glycogen synthesis is closely controlled by low molecular weight components within the cell, might explain the stepwise incorporation of ^{14}C -labelled sugars into the glycogen fraction (Fig. 4*a*) and the fluctuations in the low molecular weight intermediates, and might indirectly be the origin of the fluctuations in the mannan by influencing the intracellular levels of phosphorylated sugars and ATP.

Bud initiation is accompanied by intensive glucanase activity, concurrent with the mannanase (Maddox & Hough, 1971), but unlike mannan there was no turnover of glucan. It is possible that glucan, being situated in the inner layers of the wall (Lampen, 1968; Hough *et al.* 1971), is not lost when its bonds are broken. The linkages uncoupled during the softening of the wall which accompanies the initiation of new buds are presumably quickly made good by incorporation of new material, thus preventing any loss or turnover. New glucan is probably inserted continuously in the wall at the tip of the bud distal to the parent cell (Johnson & Gibson, 1966; Johnson, 1968).

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