Greenbaum, A. L., Slater, T. F. & Wang, D. Y. (1960). *Nature, Lond.*, **188**, 318.
Ledoux, L., Brändli, S. & De Paepe, J. C. (1958). *Nature, Lond.*, **181**, 913.
Ledoux, L., Piler, A., Vanderhaeghe, F. & Brändli, S. (1957). *Nature, Lond.*, **180**, 1049.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McDonald, M. R. (1955). In *Methods in Enzymology*, vol. 2, p. 427. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Roth, J. S. (1954). *J. biol. Chem.* **208**, 181.
Roth, J. S. (1957). *J. biol. Chem.* **227**, 591.
Slater, T. F. (1956). Ph.D. Thesis: University of London.
Slater, T. F. (1957). *Analyst*, **82**, 818.
Slater, T. F. (1961). *Biochem. J.* (in the Press).
Slater, T. F. & Planterose, D. N. (1958). *Biochem. J.* **69**, 417.
Slater, T. F. & Planterose, D. N. (1960). *Biochem. J.* **74**, 584.
Wattiaux, R. & de Duve, C. (1956). *Biochem. J.* **63**, 606.
Woodward, G. E. (1944). *J. biol. Chem.* **156**, 143.

Biochem. J. (1961) **78**, 504

Some Observations on the Form and Location of Invertase in the Yeast Cell

BY M. BURGER, E. ELIZABETH BACON AND J. S. D. BACON*
Department of Technical Microbiology, Institute of Biology, Czechoslovak Academy of Science, Prague 6, Czechoslovakia

(*Received* 13 *July* 1960)

Because it is so difficult to extract the invertase from yeast cells it has been generally supposed that the enzyme occurs in an insoluble form, requiring autolysis or the action of added enzymes for its release (cf. Gottschalk, 1958). At the same time various kinds of evidence (cf. Myrbäck & Willstaedt, 1955; Preiss, 1958) have indicated strongly that the enzyme is situated close to the cell surface and is associated with polysaccharide resembling the mannan of the cell wall.

Recently Myrbäck (1957) drew attention to the effect of ethyl acetate on baker's yeast. Cells treated overnight and washed thoroughly with water on the centrifuge lose much of their contents but retain the greater part of their invertase, even when stored for several months. Treatment with papain liberates the enzyme. Myrbäck explained this behaviour as follows: invertase is retained because it is firmly bound to insoluble structures of the cell; on plasmolysis by ethyl acetate only insignificant amounts of the enzyme are solubilized. On standing, no further enzyme is liberated because the proteinases of the yeast have been removed or destroyed during plasmolysis and washing. Only papain is known to take the place of the yeast proteinases and release the invertase.

We have described some preliminary experiments (Burger, Bacon & Bacon, 1958) which led us to a quite different conclusion, namely that the invertase is enclosed by membranes (one of which may be the cell wall) and although it is freely soluble it cannot escape until these membranes are broken by mechanical or enzymic action. In this paper we describe further experiments supporting this conclusion.

* Permanent address: Department of Biochemistry, The Macaulay Institute for Soil Research, Craigiebuckler, Aberdeen.

MATERIALS AND METHODS

Yeasts. Baker's yeast (*Saccharomyces cerevisiae*) was obtained from a factory at Libáň and brewer's yeast (*S. carlsbergensis*) from the Smichov Brewery, Prague. A strain of *S. cerevisiae*, R XII, was grown in stationary culture on 8° wort (cf. Burger, Hejmová & Kleinzeller, 1959). *S. fragilis* was grown on the same medium, usually for 24 hr. with aeration.

Snail-crop juice. Edible snails, *Helix pomatia* (obtainable from A. Gaudin, 46 Greek Street, Soho, London, W. 1), were starved for at least 24 hr. before the crop juice was collected by the method of Bawden & Pirie (1946). It was centrifuged at 1500*g* for 15 min., frozen solid or freeze-dried, and kept at −15°.

Papain. A commercial sample (Merck) and a crystalline preparation made from it, and kindly given to us by Dr V. Liebl of this Institute, were used.

Estimation of invertase activity. Convenient dilutions of the solution or suspension to be tested were added to a solution at room temperature (20°) made by dissolving 2·5 g. of sucrose in sufficient water plus 1 ml. of M-sodium acetate buffer (pH 5·0) to make a final volume of 50 ml. A sample (10 ml.) was at once pipetted into a flask containing 10 ml. of 5% (w/v) Na_2CO_3, filtered with the aid of a little Hyflo Supercel (Johns-Manville) and its optical rotation read in a 2 dm. tube in a polarimeter within 2 hr. Further samples (10 ml.) were taken and similarly treated at intervals (10 min. to 3 hr.) depending upon the activity of the solution being tested. From the fall in optical

rotation so measured the extent of hydrolysis of the sucrose was calculated.

The activity is expressed (unless otherwise stated) in units/g. of original pressed yeast, 1 unit being the amount of enzyme liberating 1 mg. of reducing sugar/min. under the above-mentioned conditions. This unit was chosen because it gave a convenient value (100–200) for the starting material; it also corresponds almost exactly to a change of the rotation read of 0·1°/hr.

Estimation of maltase activity. A similar procedure was followed, 1·0 g. of maltose being dissolved in a total of 50 ml. containing 2 ml. of 0·1M-sodium phosphate–potassium phosphate buffer (pH 6·8) and the change in optical rotation measured on 10 ml. samples added to 10 ml. of 5% (w/v) Na_2CO_3. A unit of activity is that hydrolysing 1 mg. of maltose/min. under these conditions.

Estimation of protease activity. The method of Anson (1938) was used.

Other methods. The dry weight of suspensions was measured by passing 2 ml. through a G4 sintered-glass filter, washing the residue thoroughly with water and drying it to constant weight at 105°. Kjeldahl nitrogen was measured by a micromethod, and polysaccharide content of cells by the method of Leopold & Honsig (1952–54). Paper chromatography was carried out as already described by Burger *et al.* (1959).

RESULTS

Effects of treatment with ethyl acetate on the invertase content of yeast cells

We have confirmed the observations of Myrbäck (1957) in all respects. Following his procedure exactly, preparations were obtained from *S. cerevisiae* (pressed baker's yeast, or strain RXII) having usually activities of 100–200. The amount of invertase lost during the washing process was always small, the supernatant at the first centrifuging rarely having an activity of more than 10. Table 1 describes the procedure and indicates the total losses, as shown by the activities of successive washes. About a third of the dry weight of the cells was lost during the treatment with ethyl acetate, losses being similar for the two strains of *S. cerevisiae*.

Table 1. *Losses of invertase from baker's yeast during treatment with ethyl acetate and subsequent washing*

Pressed baker's yeast (100 g.) was kneaded with 10 ml. of ethyl acetate. It at once liquefied and was shaken gently at room temperature for 15–20 hr. The suspension was dispersed in 1 l. of water and centrifuged. The precipitate was washed four times with 500–600 ml. of water and finally suspended in water to give a volume of 100 ml.

Fraction	Volume of fraction (ml.)	Activity
Ethyl acetate supernatant	920	10
First wash	620	2
Second wash	610	0
Third wash	530	0
Fourth wash	520	0
Final suspension	100	148

Chloroform was added as a preservative to the final suspensions, which were then kept at room temperature. Periodic tests on the suspensions showed that only about 10–15% became 'soluble' (i.e. failed to sediment when centrifuged at 1500*g*) even after several weeks' storage.

Strain RXII grown in the laboratory was used for many preparations because it gave a higher and more reproducible invertase activity than the commercial pressed baker's yeast. In one such preparation 15% of the invertase had become 'soluble' after storage for 3 months. An examination of shorter periods of treatment with ethyl acetate suggested that the invertase found in the ethyl acetate supernatant was not liberated instantaneously. Thus in one experiment the activities of supernatant (and sediment) after 2, 5 and 20 hr. of treatment were respectively: 4 (274), 7 (276) and 16 (270).

The only modification to Myrbäck's procedure introduced in later experiments was to shake the liquefied mixture of yeast and ethyl acetate overnight in a wrist-action shaker.

Examination under the microscope showed that the cells retained their shape but were no longer as refractive as living cells, nor did they stain with methylene blue. Viable counts showed that no living cells were present. A sample of RXII had lost all its power to ferment glucose after treatment with ethyl acetate for only 30 min. A few experiments with other yeasts showed that whereas *S. carlsbergensis* behaved similarly to *S. cerevisiae*, *S. fragilis* lost practically all its invertase from the cells during treatment with ethyl acetate.

The suspensions prepared by the use of ethyl acetate, which were always diluted so that 1 ml. was equivalent to 1 g. of original moist yeast, are hereafter referred to as Myrbäck preparations.

Liberation of invertase by mechanical disintegration

The press described by Hughes (1951) was used to disintegrate fresh yeast and Myrbäck preparations. In each case the material was frozen in the stainless-steel block and, after crushing, was washed off with a small volume of water. The suspension was centrifuged at 35 000*g* for 1 hr. in a refrigerated centrifuge (MSE Superspeed-20), giving the following separation: above a compact precipitate there was a layer of material very easily disturbed, then a transparent layer and at the top a layer of floating material, presumably lipid. With fresh yeast it was difficult to remove a sample of the clear supernatant, but from Myrbäck preparations there was much less of the floating and 'loose' layers. In both cases the clear supernatant

contained a large proportion of the total invertase of the original suspension. Tests with fresh yeast showed about 75% in the supernatant; Table 2 gives some typical results with Myrbäck preparations.

Examination under the microscope showed that the cell structure had been extensively damaged, very few cells remaining intact.

When fresh baker's yeast was disintegrated by shaking in the presence of glass beads in a 'Vibrogen Zellmühle' (E. Bühler, Tübingen, Germany), an even greater proportion of the invertase activity was found in the supernatant from high-speed centrifuging: 80% after 4 min. shaking at 50 cyc./sec. and virtually 100% after 8 min. The microscope again showed extensive cell damage.

Myrbäck preparations frozen in solid carbon dioxide and then allowed to thaw, but not crushed in the press, did not lose invertase to the suspending medium. (This observation confirms that of Myrbäck & Willstaedt, 1955.)

Table 2. *Invertase liberated by crushing of Myrbäck preparations from baker's yeast*

A sample of each preparation was crushed 1–2 hr. after the final washing. Loss of invertase to the medium when samples were kept at room temperature for 1 month was also measured.

Activity of original preparation	Activity recovered in medium after crushing	Recovery (%)	Loss on standing for 1 month
138	112	81	10
139	107	77	10
170	161	95	18
146	124	85	7

Action of papain preparations on ethyl acetate-treated yeast

When Myrbäck preparations were incubated in acetate buffer with commercial papain preparations there was a slow liberation of invertase; with some batches of papain this action was accelerated by the presence of cysteine. However, the percentage of the invertase liberated never reached 50% and the rate of liberation was not greatly increased by three- or four-fold increases in papain concentration. The crystalline-papain preparation (which had about ten times the proteolytic activity of the crude preparation), when used at concentrations at which its proteolytic potency exceeded that of the crude preparation, liberated much less invertase and in many experiments [cf. Table 3 (*b*)] no more than the control, to which only buffered cysteine had been added (i.e. 5–15%).

There seemed to be distinct differences in the susceptibility of Myrbäck preparations to papain preparations, as judged by the initial rates of liberation of invertase. Because of the relative slowness of the action of papain preparations it was difficult to test them on living yeast. In one experiment no liberation of invertase was detected after incubation for 24 hr.

Action of snail-crop juice

Snail-crop juice produced a much more rapid liberation of invertase from Myrbäck preparations than did the papain preparations and set free virtually all the enzyme (Fig. 1).

Although not dependent upon its addition, the snail-crop juice action was more rapid in the presence of cysteine. The invertase content of the

Table 3. *Liberation of invertase from ethyl acetate-treated cells in the presence of papain preparations*

A solution of the papain preparation in M-sodium acetate buffer (pH 5·0) was added to the Myrbäck preparation and the suspension left at room temperature with occasional shaking. Cysteine to a final concentration of 10 mg./ml., and chloroform as a preservative, were also added to each incubation.

(*a*) Myrbäck preparation from baker's yeast: activity 128

Time of incubation (days)	Invertase liberated: No papain	Merck papain (4·3 mg./ml.)	Merck papain (14·3 mg./ml.)	Crystalline papain (1·43 mg./ml.)
6	7	28	37	10
10	12	48	61	21
18	15	60	68	30

(*b*) Myrbäck preparation from R XII: activity 210

Time of incubation (days)	Invertase liberated: No papain	Merck papain (4·16 mg./ml.)	Merck papain (8·3 mg./ml.)	Merck papain (16·6 mg./ml.)	Crystalline papain (1·66 mg./ml.)
1	14	22	26	30	14
3	22	45	50	55	24
7	19	62	59	67	22
14	32	—	91	107	34

juice was negligible by comparison with that present in the yeast; thus a typical freeze-dried preparation had 7 units of invertase activity/g. Its proteolytic activity was too small to be measured by the method of Anson (1938); when tested at 280 times the concentration used for the papain preparation no tyrosine liberation could be detected.

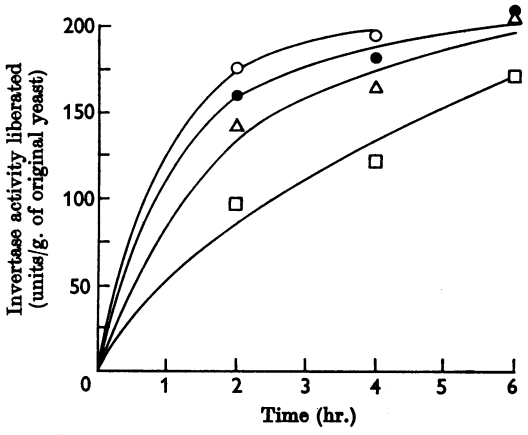

Fig. 1. Liberation of invertase from ethyl acetate-treated baker's yeast (activity 200) in the presence of various concentrations of snail-crop juice. The following quantities of freeze-dried crop juice, each dissolved in 1 ml. of water, were added to 10 ml. of unbuffered suspension and left at room temperature in the presence of chloroform: □, 2·5 mg.; △, 5 mg.; ●, 10 mg.; ○, 20 mg.

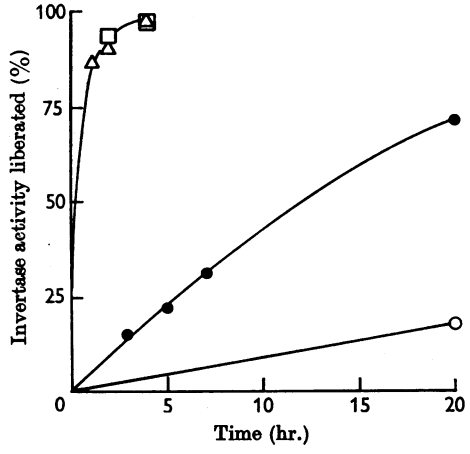

Fig. 2. Liberation of invertase by snail-crop juice from living cells of baker's yeast and *S. cerevisiae* R XII, and from Myrbäck preparations made from them. In each case 5 g. of pressed yeast or its equivalent was incubated in unbuffered suspension (total vol. 11 ml.) with 200 mg. of freeze-dried snail-crop juice at 20–22°. Living yeast: ○, baker's; ●, R XII. Myrbäck preparations: □, baker's; △, R XII.

The action of the snail enzymes on suspensions of living yeast differed in some respects from that on Myrbäck preparations. The invertase liberation was always slower (Fig. 2); the rate of liberation from R XII was very reproducible, that from baker's yeast was slower and there was variation from batch to batch. During the loss of the first half of the invertase from the cells, examination under the microscope in the presence of methylene blue

Table 4. *Changes in viable count of a yeast suspension incubated with snail-crop juice*

S. cerevisiae R XII (1 g.) was incubated in 11 ml. of water with 200 mg. of freeze-dried snail-crop juice at 30°; no buffer was added.

Time of incubation (hr.)	Invertase liberated (% of initial content)	10^7 × Viable count/ml. of suspension*: Control: no crop juice	10^7 × Viable count/ml. of suspension*: With crop juice
0	0	23·5	23·6
3	38	21·5	23·0
5	48	22·3	23·0
7	67	26·5	10·0
20	82	24·5	7·5

* Each figure is the mean of four or more counts, made from dilutions plated on 8° wort agar and incubated overnight at 30°.

Table 5. *Fermentation of various sugars by strain R XII after treatment with snail-crop juice*

Pressed yeast (2·5 g.) was incubated with 100 mg. of freeze-dried crop juice in 5 ml. of 0·1 M-phosphate buffer (pH 6·8) for 3 hr. at 30°: 57% of the invertase was liberated. A control incubation was made with no crop juice. In each case the yeast was then washed three times with water and suspended in 0·1 M-KH_2PO_4 solution (pH 5·0). These suspensions were then tested manometrically, the Q_{CO_2} being measured in the presence of 0·3% of sugar.

	Salt solution	Glucose	Sucrose	Maltose
Control	5·45	123	122·8	20·0
Treated with crop juice	4·47	106·3	110·5	20·4

Table 6. *Liberation of invertase from yeast cells treated with aqueous glycerol*

The cells (5 g. moist weight) were incubated in 10 ml. of 30% (v/v) glycerol for 18 hr. at 30°. A sample (1 ml.) of suspension was then centrifuged and the supernatant analysed (*a*). The sediment was resuspended in 10 ml. of water and immediately centrifuged; the clear supernatant was analysed (*b*).

	S. cerevisiae: R XII	*S. cerevisiae*: Baker's yeast	*S. fragilis*
Activity of intact yeast	198	96	58
Activity liberated during incubation (*a*)	12	6	47
Activity liberated after transfer to water (*b*)	48	17	5

showed little or no increase in the numbers of cells taking up the stain (less than 10 %) and the viable counts did not alter (cf. Table 4). During this stage the fermentative capacity of R XII cells was not diminished (Table 5). Later the viable count began to fall and the number of cells staining with methylene blue increased.

When the incubation was carried out in the presence of 0·2 M-lactose, to maintain the iso-osmoticity of the medium (cf. Eddy & Williamson, 1958), almost all the invertase passed into the medium although the majority of protoplasts were still intact (Fig. 3). The microscope showed spherical bodies with a boundary much less refractive than that of the original cells. Lysis was accompanied by a decrease in extinction (Weibull, 1953) and the appearance of cell debris. Protoplasts isolated after 20 hr. and washed by centrifuging in 0·2 M-lactose liberated only traces of invertase when lysed in water.

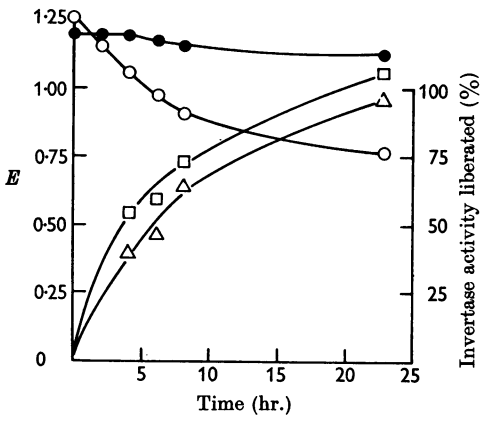

Fig. 3. Liberation of invertase from living cells of R XII by snail-crop juice in the presence of 0·2 M-lactose. Two parallel incubations were set up: each contained 2 g. pressed weight of R XII and 400 mg. of freeze-dried snail-crop juice in a total volume of 22 ml.; one incubation contained in addition 0·2 M-lactose. Both were incubated at 30°. Invertase liberation and the extinction in a 1 cm. cell of a Pulfrich photoelectric colorimeter with a yellow–green filter were measured at intervals. Extinction: ●, with lactose; ○, without lactose. Invertase: △, with lactose; □, without lactose.

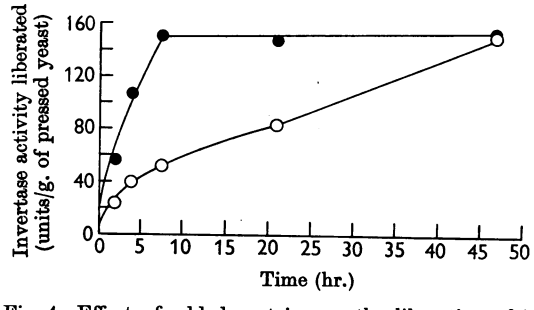

Fig. 4. Effect of added cysteine on the liberation of invertase from living yeast cells by snail-crop juice. In each case 5 g. of pressed R XII was incubated at 30° with 50 mg. of freeze-dried snail-crop juice in a total volume of 11 ml. To one incubation 70 mg. of cysteine–HCl was added. The pH of both incubations was adjusted to 5·0; after 47 hr. that with cysteine had pH 4·9, that without had pH 4·7. Invertase liberation was measured at intervals: ●, with cysteine; ○, without cysteine.

The action of the snail-crop juice on living yeast cells was also enhanced by the presence of cysteine (Fig. 4).

Liberation of invertase by osmotic shock

Living cells which had been exposed to 20–30 % (v/v) of glycerol lost an appreciable proportion of their invertase to the medium when transferred to water (Table 6). If the transfer to water was made by stages, e.g. from 20 % through 15, 10 and 5 % of glycerol, no invertase was liberated.

Tests with other concentrations of glycerol showed that less invertase was liberated at 10, 60, 80 and 100 % (v/v). Examination under the microscope showed no difference in the appearance of the cells after transfer to water and no signs of cell debris.

Table 6 shows that during exposure to 30 % (v/v) glycerol practically no invertase is liberated from *S. cerevisiae*, but almost all from *S. fragilis*.

Yields of invertase activity by various procedures

That little or no inactivation of invertase takes place during ethyl acetate treatment was suggested by the comparison of the activity of fresh samples of baker's yeast and of the preparations made from them. For example, in two such cases the activities of the intact cells were 150 and 196, and of the ethyl acetate-treated cells 168 and 201 respectively. (In the first case the maximum loss of reducing sugar by fermentation was calculated, from the carbon dioxide formed in a parallel incubation, to be equivalent to 2–3 units of activity.) This impression was confirmed by the observation that the maximum invertase activity liberated by snail-crop juice from living cells or Myrbäck preparations also approached that measured on the intact yeast. A direct comparison with R XII showed activities of 204 for the living yeast, 181 for the Myrbäck preparation and 195 for the invertase liberated from the living cells by incubation for 45 hr. with snail-crop juice. A similar correspondence was found with baker's yeast.

With *S. fragilis*, intact yeast having an activity of 48 lost 42 to the medium when treated with ethyl acetate and 51 when incubated with snail enzymes.

In no case was there any indication that the amount of invertase present was increased by the treatment being investigated.

Effect of the physiological state of Saccharomyces cerevisiae *RXII cells on their susceptibility to the action of snail-crop juice*

In the course of the experiments described above it was noticed that the responses to papain and to snail-crop juice varied from one Myrbäck preparation to another. It could also be seen by microscopical examination that some living cells, or cell 'ghosts' as the case might be, were more easily disintegrated by enzymes than others. We therefore tried the effect of aerating suspensions of RXII, with and without 7% (w/v) of galactose; the galactose was not all utilized during this period. In the absence of galactose the pressed weight of the yeast fell somewhat (1–0·83 g.) and the invertase activity/g. changed little (149–153). In the presence of galactose cell multiplication took place and the pressed weight rose (1–1·9 g.) and the total invertase also increased, but not in proportion, to give an activity of 127. The latter cells were much less susceptible to the action of snail-crop juice than the original anaerobically cultivated RXII or the cells aerated in the absence of galactose (Fig. 5); the effect of aeration with galactose was to depress the protein content and nearly to double the polysaccharide content.

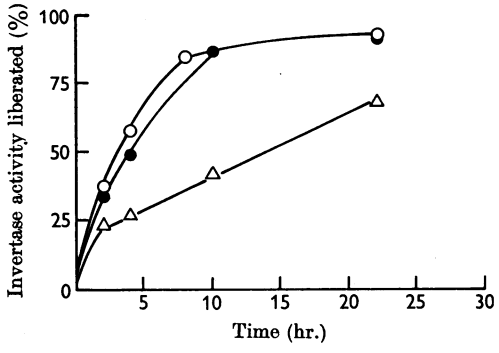

Fig. 5. Effect of aeration in the presence of galactose on the susceptibility of RXII cells to the action of snail-crop juice. Two suspensions of yeast in 0·05 M-sodium phosphate–potassium phosphate buffer (pH 6·8), one containing 7% of galactose, were divided into batches of 55 ml. each containing 0·15 g. of pressed yeast, and incubated at 30° for 16 hr. with shaking. The cells were then washed with water and batches were pooled to give 2·5 g. of pressed yeast from each suspension. This was incubated with 100 mg. of freeze-dried snail-crop juice in a total volume of 5·5 ml. A sample of the original RXII, grown in stationary culture, was tested simultaneously. ●, Stationary culture; ○, shaken culture without galactose; △, shaken culture in the presence of galactose. Other details are given in the text.

Effect of treatment with ethyl acetate on the maltase of Saccharomyces cerevisiae *RXII*

The Myrbäck preparations from RXII had high invertase activity but no maltase could be demonstrated. Examination of shorter periods of treatment with ethyl acetate showed that after 1·5 hr. all the fermentative capacity had been lost, but most of the maltase was retained. As treatment was continued maltase activity was lost by the cell fraction, but did not appear in the supernatant.

This effect seemed to be due to inactivation by the solvent. Maltase liberated from living cells by the action of snail-crop juice was stable for at least 72 hr. in 0·1 M-sodium phosphate–potassium phosphate buffer (pH 6·8), but when an excess of ethyl acetate was added all the maltase activity was lost in 3 hr. It has already been noted (Table 5) that the fermentation of maltose was not affected by a short period (3 hr.) of treatment with the juice, during which 57% of the invertase was liberated.

DISCUSSION

After yeast cells have been treated with ethyl acetate a fraction remains 'insoluble', i.e. it can easily be centrifuged at 1500*g*. After thorough washing with water a large part of the invertase of the original cells remains in this sediment, and in this sense is 'insoluble'. However, our experiments show clearly that if the sediment is crushed in the Hughes press the greater part of the invertase can no longer be centrifuged, i.e. it has become 'soluble' as a result of mechanical damage.

The invertase of the living yeast cell can also be brought into solution in this way. It therefore seems that the period of ethyl acetate treatment, although it may have encouraged autolysis and may indeed have affected the physical state of the invertase, has not brought about any decisive change in its properties, such as might be envisaged in the term 'solubilization'.

A more interesting observation is that a large proportion of the invertase may be lost from living yeast cells under the influence of snail digestive enzymes, whereas the viable count is virtually unaffected. Practically all the invertase may be 'liberated' under conditions in which considerable disintegration of cell walls takes place, but in which the escaping protoplasts remain intact.

These observations suggest an interpretation of earlier work on the 'solubilization' of yeast invertase quite different from that generally accepted; namely, that the greater part of the enzyme already exists in a soluble form outside the protoplast, but is somehow retained by the cell wall. Such an idea is supported by the evidence from various sources indicating that the enzyme is located close

to the surface of the cell. Thus Preiss (1958) has calculated from the susceptibility of the enzyme to bombardment by low-voltage electrons that in dry cells it must lie as a continuous layer, or a series of randomly disposed patches, 50 mμ below the cell surface and at no greater depth than 100 mμ. It is difficult to exclude the possibility that the invertase is present inside the protoplast, but leaks out of it while the cells are being attacked by the snail-crop juice. If this is the case, the permeability of the membrane must be selective, otherwise it is difficult to see how the cell can retain its full capacity to ferment sugars under conditions in which nearly 60% of the invertase has been lost (Table 5). For this reason we favour the hypothesis that the bulk of the invertase, wherever it may have been synthesized, comes to lie between the protoplast membrane and the cell wall.

Such a hypothesis still leaves much room for elaboration. For example, it is necessary to explain the effect of papain preparations, originally thought to deputize for autolytic proteases. Our own experiments suggest that it may not be the proteolytic activity that is responsible. There is a little β-glucosidase in commercial papain preparations and in the crystalline preparation we used. On the other hand, the rate of invertase liberation from Myrbäck preparations by snail digestive juice greatly exceeds the highest rate obtainable with the papain preparation tested. A possible explanation is that several polymers are built into the structures that enclose the enzyme. Hydrolysis of any single component would then be sufficient to liberate it, and the papain and snail enzymes may be attacking different substrates. It cannot even be assumed from the evidence available that the effect of the snail-crop juice is due to its action on the glucan of the cell wall (which it is known to attack, Holden & Tracey, 1950); it also attacks yeast mannan (Myers & Northcote, 1958) and might well attack other minor carbohydrate components. There is an interesting parallel between their actions in liberating invertase from Myrbäck preparations, and the effect of papain and snail digestive juice on fragments of yeast-cell walls, reported by Eddy (1958, especially Fig. 1).

We could find no evidence for liberation of invertase from Myrbäck preparations in the presence of high concentrations of lysozyme, which is known to attack a polysaccharide substrate.

Myrbäck (1957) showed that cysteine was necessary for the full action of papain preparations, and this seemed at first to support the idea that papain itself was acting. However, our own findings that cysteine activates the action of snail-crop juice, which apparently has relatively weak protease activity (both from our own tests and those of others: Bawden & Pirie, 1946; Myers & Northcote, 1958), suggest that some other explanation may be necessary, such as the removal of heavy-metal inhibitors, or an effect on the structure of the cell wall. In support of such an idea we have recently found that living RXII cells washed three times with cysteine solution, and then thoroughly with water, were almost as susceptible to the action of snail-crop juice as were cells tested in the presence of cysteine.

Sudden transferrence from 30% glycerol to water did not seem to lead to any permanent damage to the cells, but nevertheless about a third of the invertase was liberated. This might be explained by a temporary stretching of the cell wall, or by the formation of minor cracks in it which are not discernible under the microscope. It is certainly true that during the action of snail enzymes on Myrbäck preparations a large proportion of the invertase escapes before visible damage has been done, and among a population of living cells that has lost half its invertase it is difficult to find any cells from which the protoplast has escaped.

Apart from the irradiation studies already mentioned and the effect of pH and inhibitors on the invertase of living cells (Myrbäck & Willstaedt, 1955; Myrbäck, 1957) there are other grounds for associating invertase with the cell wall. Many attempts to purify the enzyme have led to the realization that it is closely associated with polysaccharide (cf. Sumner & O'Kane, 1946–48). Fischer & Kohtès (1951) found mannan in their purest stable preparations. Eddy & Williamson (1959) have shown that cell-wall preparations have invertase activity, although the amount held in this way is only a few per cent of the total activity of the cell. All these observations might be explained if the invertase were synthesized on the 'glucomannan side' (Northcote & Horne, 1952) of the cell wall, perhaps as part of a general laying down of material beyond the protoplast membrane. It would then be tempting to assume that the increase in invertase content shown by cells aerated for a period in sucrose solution (Neuberg & Roberts, 1946) is a consequence of the laying down of more cell-wall material. Unfortunately, we have not been able to confirm this effect with strain RXII. It may be that our failure is the result of using cells which through their previous conditions of growth are unable to respond to further feeding with carbohydrate.

There is scope for further study, particularly of the liberation of invertase from ethyl acetate-treated cells, the simpler of the two systems discussed here. It may be, as we have already suggested (Burger *et al.* 1958), that this may throw as much light upon the structure of the yeast cell as upon the nature of the invertase.

Friis & Ottolenghi (1959) have recently reported some interesting experiments with a yeast strain of defined genetic constitution. Their method of measurement of invertase differed very much from our own, and unfortunately it is not possible from their data to form any estimate of the invertase activity of their yeast on either a fresh- or dry-weight basis. They, too, found that much invertase was lost when protoplasts were separated from the cell-wall material by the action of snail digestive juice, but by direct measurement they found that intact protoplasts still showed some invertase activity. They also established that there was some invertase activity within the protoplasts that could not be measured by their method (which required the presence of 0·1 M-fructose) until they had been disrupted by repeated freezing and thawing. Cells of their strain grown on glucose–yeast water, and the protoplasts prepared from them, also had this 'internal' invertase activity, although when intact they showed no action on sucrose.

These results indicate that the situation may well be complex, and may vary from one strain of yeast to another. In discussing their results Friis & Ottolenghi (1959) state that: 'in cells which have grown in the presence of sucrose some invertase is to be found bound to the cell-wall material,' but the nature of their experiments does not justify any distinction between actual binding and physical trapping of the enzyme. It would be of particular value to know whether invertase is in fact bound to the cell-wall material in their yeast, and to be able to relate the amounts of invertase concerned to the total content of the enzyme in the cells of a typical commercial baker's yeast.

SUMMARY

1. Yeast cells (*Saccharomyces cerevisiae*, *S. carlsbergensis*), when treated with ethyl acetate and thoroughly washed with water, retain the greater part of their invertase, but *S. fragilis* loses it to the suspending medium.

2. When ethyl acetate-treated cells were disrupted in the Hughes (1951) press almost the whole of their content of invertase was liberated in a soluble form. The enzyme was also wholly liberated by the action of crop juice of *Helix pomatia*, and partly liberated by the action of papain preparations.

3. Snail-crop juice also liberated the greater part of the invertase from living yeast cells, even under conditions in which the protoplasts remained intact.

4. The action of both enzyme preparations in liberating invertase from living or ethyl acetate-treated cells was enhanced by the presence of cysteine.

5. It is concluded that the invertase of the yeast cell occurs in a soluble form in a region of the cell outside the protoplast but inside the cell wall, and not, as previously supposed, in combination with insoluble cell structures.

This work was done with the able assistance of Mrs L. Hejmová and Miss E. Masnerová. Two of us (E. E. B. and J. S. D. B.) wish particularly to thank Academician I. Málek, Director of the Institute, and the Czechoslovak Academy of Science, for most generous hospitality which made our participation in this work possible. We wish also to thank Dr D. H. Northcote and Professor A. A. Eddy for helpful discussions of some of the problems raised.

REFERENCES

Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Bawden, F. C. & Pirie, N. W. (1946) *Brit. J. exp. Path.* **27**, 81.
Burger, M., Bacon, E. E. & Bacon, J. S. D. (1958). *Nature, Lond.*, **182**, 1508.
Burger, M., Hejmová, L. & Kleinzeller, A. (1959). *Biochem. J.* **71**, 233.
Eddy, A. A. (1958). *Proc. Roy. Soc.* B, **149**, 425.
Eddy, A. A. & Williamson, D. H. (1958). *Nature, Lond.*, **179**, 1252.
Eddy, A. A. & Williamson, D. H. (1959). *Nature, Lond.*, **183**, 1101.
Fischer, E. H. & Kohtès, L. (1951). *Helv. chim. acta*, **34**, 1123.
Friis, J. & Ottolenghi, P. (1959). *C.R. Lab. Carlsberg*, **31**, 259.
Gottschalk, A. (1958). In *Encyclopaedia of Plant Physiology*, vol. 6, p. 87. Ed. by Ruhland, W. Berlin: Springer-Verlag.
Holden, M. & Tracey, M. V. (1950). *Biochem. J.* **47**, 407.
Hughes, D. E. (1951). *Brit. J. exp Path.* **32**, 97.
Leopold, H. & Honsig, E. (1952–54). *Zbl. Bakt.* **107**, 449.
Myers, F. L. & Northcote, D. H. (1958). *J. exp. Biol.* **35**, 639.
Myrbäck, K. (1957). *Arch. Biochem. Biophys.* **69**, 138.
Myrbäck, K. & Willstaedt, E. (1955). *Ark. Kemi*, **8**, 367.
Neuberg, C. & Roberts I. S. (1946). *Sci. Rep. Ser. Sug. Res. Fdn, N.Y.*, no. 4.
Northcote, D. H. & Horne, R. W. (1952). *Biochem. J.* **51**, 232.
Preiss, J. W. (1958). *Arch. Biochem. Biophys.* **75**, 186.
Sumner, J. B. & O'Kane, D. J. (1946–48). *Enzymologia*, **12**, 251.
Weibull, C. (1953). *J. Bact.* **66**, 688.