LXXVI. GALACTOZYMASE CONSIDERED AS AN ADAPTIVE ENZYME.

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PREVIOUS studies from this department on adaptive enzymes have been concerned with hydrogenlyases. It has been shown that the formation of these enzymes in the Bacteriaceae is controlled by the medium in which the cell is grown, one factor being the presence of the substrate, *e.g.* formate in the case of formic hydrogenlyase, another being some substance in broth which so stabilises the enzyme that it is active in washed suspensions [Stephenson and Stickland, 1932; 1933; Yudkin, 1932]. The mechanism by which the medium conditions the formation or stabilisation of enzymes was discussed by Yudkin [1932], who postulated two possibilities, either natural selection or chemical adaptation, *i.e.* direct chemical action of the substrate on the cell. He concluded, chiefly from theoretical considerations, that the production of formic hydrogenlyase by *Bact. coli* was of the nature of a chemical adaptation. This was later proved by Stephenson and Stickland [1933], who showed that natural selection was not operating, since the enzyme was formed in the absence of cell multiplication. In the present study we present some facts connected with the formation of galactozymase, the enzyme complex concerned with the fermentation of galactose by *S. cerevisiae*.

The literature already contains material on this subject. Dienert [1900] first recorded the adaptive nature of galactozymase by showing that certain strains of yeast, which at first are unable to grow in media where galactose is the sole carbohydrate, become able to do so if previously grown for a few weeks on a mixture of glucose and galactose. In such cases the same result is achieved by lactose or melibiose, doubtless because these sugars give galactose on hydrolysis. Yeast grown on glucose alone is unable to ferment added galactose, whilst yeast grown on galactose or on a mixture of glucose and galactose, when subsequently placed in contact with galactose, causes an immediate fermentation of that sugar, *i.e.* is adapted to galactose. Non-adapted yeast if left in contact with galactose for 24 hours or longer begins a slow fermentation; after this is complete, a further addition of galactose is fermented immediately, *i.e.* the yeast has become adapted. Moreover, adapted yeast, if allowed to ferment glucose, showed subsequently a diminished power to ferment galactose, that is, adaptation is partly reversible. This observation was confirmed by Euler and Johansson [1912] and Euler and Lövgren [1925] and by Söhngen and Coolhaas [1924], and contradicted by Abderhalden [1926, 2].

These experiments of Dienert, though highly suggestive, are difficult to interpret owing principally to their non-quantitative nature, rates of fermentation, quantity of yeast and change in cell numbers not being recorded.

Slator [1908] showed that only certain strains of yeast can be adapted to galactose. He was of opinion that the fermentation of galactose does not occur through glucose or through a form common to both sugars, since unadapted yeast when added to adapted yeast does not increase the rate of fermentation of galactose.

Harden and Norris [1910] found galactozymase in the press juice from adapted but not from unadapted yeast, thus disposing of the idea that adaptation consists in increasing the permeability of the cell to galactose. They also showed that the rôle of phosphate in galactose fermentation is similar to that in glucose fermentation.

Abderhalden [1926, 2] showed that dried adapted yeast retains its fermentative power for long periods; he claimed also to have adapted dried yeast, but this was probably due to the adaptation of surviving cells which multiplied during the process. He also showed [1926, 1] that adaptation is not due to the formation in the medium of substances accelerating the fermentation of galactose, whilst Euler and Jansson [1927] excluded the possibility that adaptation is concerned with the formation or modification of cozymase.

Evidence on the connection between adaptation and viability is conflicting. Söhngen and Coolhaas [1924] carried out adaptation at 30° and measured fermentation at 38° in order to avoid cell multiplication during fermentation. They found that the rate of fermentation was proportional to the number of new cells formed and concluded that the new cells formed during adaptation are alone responsible for the fermentation. They found that, when adapted yeast ferments glucose, the adaptation is partially reversed, but that this process is independent of multiplication. No adaptation occurred in two weeks at 0°. Euler and Johansson [1912] stated that adaptation occurs only in the presence of galactose and asparagine and certain salts; later Euler and Nilsson [1925] found that, though adaptation does not occur in galactose and water, it does so if the "Z" factor is added. This evidence is on the whole in favour of adaptation being associated with growth. Abderhalden on the other hand [1926, 1] supported Dienert in showing that adaptation occurs in an aqueous solution of galactose and stated on microscopic evidence that growth did not occur.

The evidence obtained by adapting yeast in the presence of growth inhibitors is untrustworthy. Euler and Nilsson [1925] tried to adapt yeast in the presence of about 0.5 % phenol, and found that after the removal of the phenol the yeast fermented galactose after a latent period of about 15 hours. In the absence of sterility tests or of viable cell counts this evidence is inconclusive. Similar experiments were done with alcohol-treated dried yeast. This work was criticised by Söhngen and Coolhaas [1926], who showed that, in the conditions of Euler and Nilsson's experiments, cell multiplication occurs when the yeast poisoned by phenol or by alcohol is subsequently placed in contact with galactose. Euler and Jansson [1927] accepted the criticisms of Söhngen and Coolhaas, and modified their original experiment by carrying out the fermentation in the presence of phenol, with negative results. They also attempted to dissociate growth and adaptation by carrying out the latter process at 38°; at this temperature neither growth nor adaptation occurred.

The following work was done to determine the exact conditions in which galactozymase is formed and the relation between the formation and activity of the enzyme complex with the multiplication and viability of the cell.

TECHNIQUE.

The yeast used was a pure strain of *S. cerevisiae* obtained from the National Collection of Type Cultures. It was grown in Roux bottles on a tryptic digest of caseinogen of half the strength used for bacteria with 1 % of glucose (or galactose) and at a p_H of about 6.0. The bottles were incubated at 28°; in the earlier experiments the growth period was 2–3 days, but later this was extended to 6 days for reasons given on p. 509. The yeast was centrifuged and washed twice with sterile

water; the crop from 1 l. was made up to about 15 ml. The fermentative power of the suspension was measured by the rate of carbon dioxide evolution in a Barcroft manometer. In the right-hand cup were placed 1 ml. of phosphate buffer p_H 6.0, 1 ml. of 5% sugar solution and 1 ml. of yeast suspension; in the left-hand cup the sugar solution was replaced by water. The apparatus was evacuated twice and filled with nitrogen previously passed over heated copper. The fermentations were done at 30°. Sterile precautions were observed throughout except in the manometers.

All samples of galactose (including Kahlbaum's) tested by us contained enough glucose or other fermentable sugar to give a measurable fermentation with untrained yeast in the Barcroft apparatus. We purified our galactose by a modification of the method by which Somogyi [1927] removed glucose from blood by adsorption by yeast. 100 g. of baker's yeast were washed in 1 l. of distilled water and centrifuged; this process was performed 4 times. The washed yeast was then suspended in 100 ml. of 20% galactose, made up to 200 ml., left for 10 min. and centrifuged; 100 ml. of the supernatant fluid were then treated with 50 g. of washed yeast as before and the process exactly repeated. 100 ml. of the final supernatant fluid were treated with 8 ml. of "colloidal iron" and filtered twice through kieselguhr. The final solution contained between 4 and 5% of galactose, and 1 ml. of this solution was used in the Barcroft cup for each fermentation experiment.

The total counts were made after suitable dilution by means of a Zeiss Thoma slide as used for blood counts. The viable counts were made by the roll tube method as described by Wilson for bacteria [1922]. The broth used for the agar medium was identical with that used for growing the yeast, with about 0.05% of marmite (wet weight) added in order to increase the size of the colonies. Incubation was at 28° and the counts were made at the end of 3 or 4 days.

Preliminary experiments.

In accordance with previous observers we found that our yeast when grown on glucose broth failed to ferment galactose; when grown on galactose broth it fermented both sugars. For example, a suspension of yeast grown on glucose broth fermented galactose at a rate of 280 μ l./hr. and failed to ferment galactose at a measurable rate. Another sample grown on galactose broth fermented glucose at 830 μ l./hr. and galactose at 490 μ l./hr. The ratio of the rates of galactose to glucose fermentation was thus about 0.6 and this ratio was generally obtained. Previous observers have obtained ratios from 1.2 [Harden and Norris, 1910] to 1.6 [Dienert, 1900; Willstätter and Sobotka, 1922], but in these cases the conditions of growth differed from ours.

Conditions affecting the formation of galactozymase.

The yeast from 900 ml. of glucose medium was grown, centrifuged and washed in the usual way, and the suspension made up to 15 ml. Into 4 graduated centrifuge-tubes was put 1 ml. of yeast suspension together with galactose and other reagents (see Table I).

Table I.

Yeast suspension ml.	5% galactose ml.	Inorganic medium ml.	1% asparagine ml.	Ringer's solution ml.	Phosphate buffer p_H 6.0 ml.	Water ml.	Fermentation rate μ l. CO ₂ /hr.
1	1	1	0	0	0	2	104
1	1	1	1	0	0	1	100
1	1	0	0	1	0	1	117
1	1	0	0	0	1	1	115

The inorganic medium contained ammonia as source of nitrogen [Stephenson, 1930, p. 275, vi]. Each centrifuge-tube was incubated for 2 days, centrifuged and the yeast washed and made up to 3 ml.; the fermentation rate of the yeast was then determined. It appears from these results that the nitrogenous constituents play no part in the adaptation process, the presence of galactose alone being the determining factor. The effect of temperature on adaptation is shown in Table II.

Table II.

Temperature during adaptation °C.	Rate of fermentation of galactose $\mu\text{l. CO}_2/\text{hr.}$
0	0
20	18
30	63
37	60

Adaptation occurs independently of oxygen. A yeast suspension was treated with galactose for 2 days; in the first case the reaction was carried out in a centrifuge-tube as usual; in the second case air was bubbled continuously through the centrifuge-tube; in the third case the conditions were anaerobic. The subsequent rates of galactose fermentation ($\mu\text{l. CO}_2/\text{hr.}$) were 230, 250 and 200 respectively.

Association of enzyme production and growth.

From the previous experiment it is seen that in the presence of galactose the washed yeast suspension acquired the enzyme as well in water or Ringer's solution as in the presence of a source of nitrogen. This raises the question whether yeast must grow in order to produce the enzyme. We attacked this problem by several methods; in the first place by making a series of measurements of enzyme activity associated with total cell counts. In each of 6 graduated centrifuge-tubes were placed with sterile precautions 3 ml. of 5% galactose solution, 3 ml. phosphate buffer at p_{H} 6.0 and 2 ml. of yeast suspension; the tubes were incubated at 28°. At intervals of 24 hours one tube was withdrawn, centrifuged and the yeast washed and made up to 3 ml.; 0.5 ml. was withdrawn for counting and the remainder used for the determination of fermentation rates. From Fig. 1 it is seen that the total number of cells remains approximately constant whilst the enzyme activity rises to a peak on the second day and then falls off. In a similar experiment (Fig. 2) the galactozymase rises to a peak on the first day while the cell count remains constant, after which the activity falls off while the cell count rises. From these two experiments it appears that enzyme formation is not necessarily accompanied by cell multiplication. These results are however not perfectly conclusive. In the first place, the proportion of viable to total cells in different experiments may vary from 5 to 50%. Where the ratio is low it is possible that the viable cells may increase 100% whilst causing an increase of only 5% on the total, the increase thus falling within the experimental error; for this reason, in most of our experiments we employed a viable count. During many of the experiments described later we found a definite increase in viable cells during adaptation, whilst in others they remained constant and sometimes decreased. Finally we chanced on the explanation for these contradictory results. In our earlier experiments the yeast was grown for 2 or 3 days, by which time the glucose in the medium was not completely fermented, and the yeast therefore in a good state of nutrition. If the growth period is prolonged to 6 days the glucose is exhausted and the yeast deprived of much of its store of nutrients; in these circumstances adaptation can

Fig. 1. ●—● Enzyme activity; ○—○ total cell count.

Fig. 2. ●—● Enzyme activity; ■—■ total cell count.

Table III. *Relation between adaptation and growth.*

Exp.	Before adaptation	After adaptation	Increase %
	Viable cells/ml. $\times 10^7$.		
1	4.5	2.7	- 40
2	3.7	4.0	+ 8.1
3	5.17	5.4	+ 3.8
	Total cells/ml. $\times 10^7$.		
4	17.8	17.9	+ 0.6

be obtained repeatedly without any increase in viable count (see Table III). In order to meet the objection that the cell multiplication is actually occurring but balanced by an equal number of deaths a total count was also included.

These experiments prove that natural selection plays no part in the formation of galactozymase, which furnishes another example of chemical adaptation.

Association of enzyme formation and viability.

The next point we tried to determine was whether enzyme formation can occur in "dead," by which we mean non-viable, cells. We first attacked this problem by poisoning a culture to the point of complete sterility with various poisons, and then subjecting it to the adaptive process in the usual way. In these experiments we obtained negative results throughout. The following poisons were tried: saturated solutions of toluene and of chloroform, 2% propyl alcohol, mercuric chloride $M/500$ to $M/2000$ and 2% cyclohexanol. It was found that, where enzyme formation occurred, subcultures of the suspension into glucose broth invariably grew, and where complete sterility occurred, no enzyme formation took place.¹

A statistical study of viability and galactozymase activity was next undertaken by the use of graded doses of ultraviolet light. The first step was to determine the relation between the activity of galactozymase once formed and the number of viable cells. Yeast from 750 ml. of medium was centrifuged and washed, made up to 14 ml. and transferred to a sterile bottle of 150 ml. capacity containing about 50 glass balls about the size of a pea; the bottle was closed with a glass stopper, sealed with hot paraffin wax and shaken on a mechanical shaker for 2 hours. If (as in Exp. 2, Table V) the shaking is prolonged to 12 hours, the proportion of viable to total cells becomes decreased. After shaking, 6 ml. of yeast suspension + 1.5 ml. of phosphate buffer p_H 6.0 + 1.5 ml. of 5% galactose solution were placed in each of two graduated centrifuge-tubes and incubated at 28° for 2 days. The tubes were then centrifuged and the yeast washed once and made up to 6 ml.; 1 ml. was removed and diluted for counting and 3 ml. for fermentation, and the remainder exposed in a double quartz tube with a water jacket 6 in. from a mercury lamp; details of this procedure are given in a previous paper [Cook and Stephenson, 1928]. Two exposures each of 5 min. were given; after each exposure 1 ml. was withdrawn for a viable count and 3 ml. for fermentation (Table IV).

Table IV. *Effect of irradiation by ultraviolet light on viability and on galactozymase.*

Time of exposure, min.	0	5	10
Total count, cells/ml.	17×10^7	17×10^7	17×10^7
Viable count, cells/ml.	(a) 3.6×10^7	2.3×10^8	0
Rate of fermentation of galactose, μ l. CO_2 /hr.	(R) 259	134	60
	(R/a) 1.98×10^{-4}	5.83×10^{-2}	∞

From this it is seen that with an initial ratio of viable to total cells of 0.47 the fermentation rate is 259; when 1 in 10,000 only of these cells is viable the fermentation rate is reduced to about one-half, and with complete sterility the rate is still one-fifth of the original. Hence it is clear that the action of

¹ A similar result has been recorded with the formation of the adaptive bacterial enzyme decomposing the specific polysaccharide of the Type III Pneumococcus [Dubos, 1935]. Here the question of cell multiplication is undecided, but no enzyme formation occurs in the presence of antiseptics.

galactozymase, once formed, is independent of viability. Experiments with glucozymase gave similar results.

The relation between the formation of galactozymase and viability was next attacked. Two possibilities occurred to us. The formation of the enzyme may be conditioned by the number of viable cells present before adaptation, or by the number present after adaptation, supposing—as in our earlier experiments—some increase or decrease to have occurred.

In order to test these possibilities yeast suspension was made in the usual way and total and viable counts were made. One portion was then exposed to ultraviolet light for 5 min., and another for 10 min. A sample from each was then withdrawn for a viable count and the remainder incubated with buffer and galactose for 2 days. A second viable count was then made and a fermentation rate with galactose determined. The results of two experiments are given in Table V.

Table V.

Cells/ml. $\times 10^6$		Time of exposure (min.)			
		0	5	10	
Exp. 1					
Total before adaptation		190	—	—	
Viable before adaptation	(a)	91	9.1	0.59	
Viable after adaptation	(b)	110	13.4	3.2	
Increase of viable during adaptation	(c)	19	4.3	2.6	
Velocity of galactose fermentation, $\mu\text{l. CO}_2/\text{hr.}$	(R)	209	198	127	
	(R/a)	2.3	21.7	215	
	(R/b)	1.9	14.8	39.6	
	(R/c)	11	46	49	
Cells/ml. $\times 10^6$		Time of exposure (min.)			
		0	3	6	10
Exp. 2					
Total before adaptation		147	—	—	—
Viable before adaptation	(a)	8.4	5.3	0.165	0.00036
Viable after adaptation	(b)	55	29.5	2.57	0.00085
Increase of viable during adaptation	(c)	47	24.2	2.41	0.00081
Velocity of galactose fermentation, $\mu\text{l. CO}_2/\text{hr.}$	(R)	243	215	51	0
	(R/a)	29	40.6	315	—
	(R/b)	4.4	7.3	19.9	—
	(R/c)	5.2	8.9	24	—

Though we do not advance these experiments as proof that adaptation is independent of viability, we think the variability of the ratios (R/a , R/b and R/c) strongly suggests this conclusion. Nevertheless this variability is of a totally different order from that obtained in Table IV, where viability and enzyme activity are in question and where indeed the ratio viable cells/enzyme activity varies from 1.98×10^{-4} to ∞ .

Non-adaptive nature of glucozymase.

Since yeast is always grown on media containing glucose or sugars giving glucose on hydrolysis it seemed possible that glucozymase itself might be adaptive. *S. cerevisiae* was accordingly grown for four subcultivations on broth with purified galactose free from glucose. At the end its fermentative power on glucose was unimpaired, hence glucozymase in this strain is not an adaptive enzyme.

Influence of glucose on galactozymase.

Owing to conflicting reports in the literature, we investigated the influence of glucose on galactozymase. Tubes of yeast were adapted in galactose and the

fermentation rate determined on successive days. On the second day 6 tubes were centrifuged and the yeast washed and replaced as follows:

- 2 with fresh galactose;
- 2 with water;
- 2 with glucose.

The fermentation rates of all were determined on the two successive days; the results are shown in Fig. 3. It is seen that the replacement of the galactose by glucose in the training tubes causes a disappearance of galactozymase which is almost complete in 2 days. Fresh galactose, on the contrary, causes a slight increase in galactozymase, which again falls off, whilst, when galactose is replaced

Fig. 3. ●—● Adaptation in galactose; ▲---▲ fresh galactose added; ■····■ galactose replaced by water; ○---○ galactose replaced by glucose.

Fig. 4. ●—● Adaptation in galactose; ●---▲ replacement of galactose by glucose; ■····■ replacement of glucose by galactose.

by water, the course of the fermentation closely follows the course of the tubes where the original galactose is undisturbed. Fig. 4 gives a similar experiment showing a disappearance of galactozymase on treatment of the yeast with glucose and its reappearance on the renewal of galactose.

SUMMARY.

1. In confirmation of Dienert and of others we find that in *S. cerevisiae* galactozymase is an adaptive enzyme.
2. In confirmation of Dienert and of Abderhalden adaptation was proved to occur in aqueous solutions of galactose.
3. Adaptation can occur without cell multiplication. Thus, as in the case of formic hydrogenlyase, adaptation occurs, not as a result of natural selection, but as a response of the cell to its chemical environment.
4. We have so far been unable to obtain adaptation in sterile cultures, although a study of total and viable counts suggests that it is not viable cells alone which are capable of adaptation.
5. The activity of galactozymase and of glucozymase is independent of cell viability.
6. In confirmation and extension of earlier work we find that adapted cells lose their galactozymase completely after fermenting glucose and regain it in the presence of galactose.

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