The Pathway of the Adaptive Fermentation of Galactose by Yeast

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Certain yeasts can ferment galactose by the formation of an adaptive 'galactozymase' when placed in aqueous solutions of galactose and in the apparent absence of cell division (Stephenson & Yudkin, 1936; Spiegelman, 1945). Though much work has been done on the mechanism of this adaptation (Spiegelman, 1947) little knowledge has so far been obtained concerning the actual enzymic changes involved or of the intermediates formed.

Kosterlitz (1943b), who has reviewed the information available, showed that a maceration juice prepared from galactose-adapted yeast would ferment both α -glucose-1-phosphate and α -galactose-1-phosphate in a similar manner; he suggested that α -galactose-1-phosphate played the same role in galactose fermentation as did α -glucose-1-phosphate and glucose-6-phosphate in glucose fermentation. Working with dialyzed yeast extracts, Kosterlitz showed that 27% of the α -galactose-1-phosphate added was converted into a difficultly hydrolyzable ester. In the same time, about 90% of α -glucose-1-phosphate was converted to glucose-6-phosphate. To explain these results, he suggested that at least two new enzymes were involved in galactose fermentation: one converting galactose to α -galactose-1-phosphate, and a second converting this ester into α -glucose-1-phosphate which is then transformed into the Robison ester.

The work presented in this paper describes the preparation of an extract from galactose-adapted yeast and the formation of an easily hydrolyzable phosphoric ester from galactose and adenosinetriphosphate in the presence of this extract. This ester has been identified as α -galactose-1-phosphate. Some evidence is also presented in favour of the view that galactose-1-phosphate is further transformed into glucose-6-phosphate.

EXPERIMENTAL

Organisms, growth media and preparation of extracts. The work was carried out with a strain of Dutch top yeast (Cat. no. 174) obtained from the Carlsberg Laboratories. The yeast was grown in Roux bottles, each of which contained 150 ml. of the following medium: 0.1% $\text{NH}_4\text{H}_2\text{PO}_4$; 0.1% $(\text{NH}_4)_2\text{HPO}_4$; 0.2% KH_2PO_4 ; 0.2% Difco Yeast Extract; 2% galactose; 1% of a trace-element solution. The trace-element solution contained, per litre: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20.0 g.;

NaCl , 1.0 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{MnSO}_4 \cdot 3\text{H}_2\text{O}$, 0.5 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05 g.; 0.1N- H_2SO_4 , 10 ml.

The medium was sterilized and inoculated from a 24 hr. culture of the yeast in the above medium at 25°. After 36 hr. at 25° the yeast was harvested, washed on the centrifuge with 0.05M- NaH_2PO_4 , and made into a thick suspension in a solution containing 0.1% galactose and 0.01% neutralized Na_2S . The suspension was then shaken with glass beads for 15 min. on a Mickle disintegrator (Mickle, 1948). It was found necessary to carry out the disintegration under anaerobic conditions in order to obtain fully active extracts: such conditions were obtained by the addition of 0.01% neutralized Na_2S to the suspension, and by filling the gas space in the vessels of the disintegrator with H_2 . The glass beads were filtered off on a coarse sintered-glass filter funnel, and the filtrate centrifuged at 3000 r.p.m. for 15 min. The cell-free supernatant fluid was used for the experiments.

Reagents and methods. The adenosinetriphosphate (ATP) used was made from a commercial preparation of the barium salt supplied by Boots Ltd. This was purified by dissolving it in 0.2N- HNO_3 until the solution was just acid to Congo red, and then by following the final stages of the preparation described by Needham (1942). Galactose-6-phosphate was prepared according to the method of Levene & Raymond (1931), and α -glucose-1-phosphate by the method of Hanes (1940). Glucose and galactose solutions were prepared from the pure bacteriological sugars supplied by Kerfoot Ltd.

Acid production. The phosphorylation of hexose by ATP in the 1 or 6 position results in an increase in acidity of the reaction mixture. Phosphorylation can therefore be followed manometrically by measuring the CO_2 evolved when the reaction is carried out in a bicarbonate- CO_2 buffer system (Colowick & Kalckar, 1943). Warburg manometers were used for this purpose.

Reducing sugar was estimated by the method of Somogyi (1945). Samples were first deproteinized by means of ZnSO_4 and $\text{Ba}(\text{OH})_2$, which also removes any phosphorylated hexose esters. Fructose was determined by the method of Roe (1934), and pentose by the method of Meijbaum (1939). The distribution of P in the various fractions was determined by estimation of orthophosphate by the Fiske & Subbarow method (1925), as follows: direct estimation, $\text{P}_{0\text{ min.}}$ = ortho-P; after wet ashing = total P; after hydrolysis in N-HCl at 100° for 7 min., $\text{P}_{7\text{ min.}}$; by calculation, $\text{P}_{7\text{ min.}} - \text{P}_{0\text{ min.}}$ = acid-labile P; total P - $\text{P}_{7\text{ min.}}$ = acid-stable P.

Separation of the Ba salts of the phosphoric esters formed during the reaction was carried out according to the procedure of Umbreit, Burris & Stauffer (1945).

Differential analysis of 'glucose' and 'galactose' by fermentation was carried out by a modification of the method of Harding & Grant (1931-2). Dutch top yeast was used in

place of baker's yeast, and the disappearance of sugar was followed manometrically by the anaerobic CO_2 evolution. When this was complete, the residual sugar was measured by the method of Somogyi (1945).

RESULTS

Preliminary experiments with the crude extract. Neither glucose nor galactose was fermented by the crude extract, even after the addition of boiled yeast extract, hexosediphosphate, acetaldehyde and ATP, singly or in combination; hexosediphosphate itself was not fermented. The ability of the extract to phosphorylate glucose or galactose in the presence of ATP was tested by following the increase in acidity in bicarbonate buffer, and the results were checked at the end of the reaction by measurement of the free sugar disappearing. The results are shown in Table 1.

Table 1. *Effect of adenosinetriphosphate on the increase in acidity (CO_2 output) and sugar disappearance on addition of glucose and galactose to extracts prepared from normal and galactose-adapted Dutch top yeast*

(The manometers contained—Main chamber: extract, 0.6 ml.; 0.5M-NaF, 0.2 ml.; 0.12M- NaHCO_3 , 0.5 ml.; 0.1M- MgCl_2 , 0.2 ml. Side bulb: 0.12M- NaHCO_3 , 0.3 ml.; and, where added, glucose or galactose (20 mg./ml.) 0.2 ml.; ATP (2.5 mg. P_7 min./ml.), 0.4 ml. Gas phase: $\text{N}_2 + 5\%$ CO_2 ; temp. 25° . Incubation time: 30 min., after which the free sugar was measured on a 1 ml. sample.)

	Crude extract		Purified extract	
	Normal	Galactose adapted	Normal	Galactose adapted
CO_2 output ($\mu\text{mol.}$)				
Control	0.18	0.18	0.09	0.09
Control + ATP	7.06	7.59	2.05	1.61
Glucose	0.80	0.98	0.09	0.18
Glucose + ATP	10.52	11.68	8.72	8.39
Galactose	0.01	0.00	0.00	0.09
Galactose + ATP	6.97	12.91	2.18	8.35
Sugar disappearance ($\mu\text{mol.}$)				
Glucose	0.00	0.56	0.00	0.00
Glucose + ATP	8.78	9.12	12.92	14.21
Galactose	0.00	0.00	0.00	0.00
Galactose + ATP	0.22	10.00	0.00	13.98

There is no significant CO_2 output or sugar disappearance in the absence of ATP, whereas in its presence there is a considerable CO_2 production in the control, which is significantly increased in presence of glucose or galactose in the case of the extract prepared from galactose-adapted yeast. In the case of the extract from yeast grown in glucose there is an increased CO_2 production over the control only when glucose is added. The increased CO_2 production after the addition of glucose or galactose can be correlated with the disappearance of sugar in

both instances. The large CO_2 output in the control was undesirable, and to minimize this it was necessary to effect some purification of the enzymes concerned.

Purification of the extract. Attempts to remove interfering sugars by dialysis resulted in inactivation of the enzyme system attacking galactose. Even dialysis overnight at 0° against an aqueous solution containing 1% galactose and 0.1% neutralized Na_2S resulted in a complete loss of activity, which could not be restored by the addition of cysteine or of a boiled extract from either normal or galactose-adapted yeast, singly or together. Hexokinase was not inactivated by this dialysis.

The extract was, therefore, fractionated by precipitation, and the following procedure was ultimately adopted. The extract was cooled to 0° and the pH brought to 4.4 by the addition of M-acetate buffer. The precipitate was separated on the centrifuge and discarded. The supernatant fluid was adjusted to pH 7.5 with 0.1N-NaOH and solid $(\text{NH}_4)_2\text{SO}_4$ added to bring the final concentration to 75% saturation. After centrifuging, the precipitate was dissolved in a small volume of distilled water and used for the following experiments.

The CO_2 output in absence of sugar was largely eliminated by the purification, as can be seen from Table 1. The increased CO_2 production, after the addition of glucose or galactose, can again be correlated with the disappearance of sugar.

Activation of the enzyme phosphorylating galactose. During the purification process the enzyme lost some activity, which was largely restored by the addition of Mg^{++} , a further increase in activity resulting on the addition of cysteine (Fig. 1). Thus, this system resembles all known phosphokinases in requiring Mg^{++} , and in being activated by thiol compounds.

The presence of phosphoglucomutase and phosphohexose-isomerase in the extract. The presence of these enzymes in the extract was tested as follows. Manometers were set up containing—Main chamber: glucose-1-phosphate (4.0 mg. P_7 min./ml.), 0.7 ml.; 0.5 M-NaF, 0.3 ml.; 0.12M- NaHCO_3 , 0.4 ml.; 0.1M- MgCl_2 , 0.1 ml.; 0.25M-cysteine, 0.1 ml. Side bulb: 0.1M- NaHCO_3 , 0.25 ml.; extract, 0.75 ml. (boiled extract, 0.75 ml. in control).

The manometers were filled with a CO_2/N_2 mixture containing 5% CO_2 , and the contents of the side bulbs tipped into the main cup after equilibration. After shaking for 90 min., 2 ml. of the contents were taken for analysis as follows: 1 ml. of 10% trichloroacetic acid was added to each sample, and the precipitate centrifuged and washed with water. The supernatant fluid and washings were combined, brought to pH 7.5 with 10% NaOH, and diluted to 10 ml. Determinations were then made of total P, acid-labile P (corresponding to glucose-1-phosphate P), and of the fructose content from which the P present as fructose-6-phosphate was calculated. Aldose-6-phosphate P was then calculated as (acid-stable P — fructose-6-phosphate P).

In the control experiment with the boiled extract, 92% of the glucose-1-phosphate was unchanged at the end of the experiment; with the active extract an equilibrium mixture was obtained consisting of 15%

glucose-1-phosphate, 26 % fructose-6-phosphate and 59 % aldose-6-phosphate. Thus phosphoglucumutase and phosphohexoseisomerase were present in the extract.

Fig. 1. Activation of the galactose-phosphorylating system in an extract prepared from galactose-adapted Dutch top yeast. The manometers contained—Main chamber: extract, 0.6 ml.; 0.5 M-NaF, 0.2 ml.; 0.12 M-NaHCO₃, 0.5 ml.; and, where added, 0.1 M-MgCl₂, 0.1 ml.; 0.25 M-cysteine, 0.1 ml. Contents made up to 1.5 ml. with distilled water. Side bulb: 0.1 M-NaHCO₃, 0.3 ml.; and, where added, ATP (3.0 mg. P_{7 min.}), 0.4 ml.; galactose (20 mg./ml.), 0.2 ml. Contents made up to 0.9 ml. with distilled water. Gas phase: 95 % N₂ + 5 % CO₂; temp. 25°. —, mixtures containing galactose; ----, mixtures without galactose; ●, ATP, Mg⁺⁺ and cysteine; ○, ATP and Mg⁺⁺; + ATP only; △, Mg⁺⁺ and cysteine.

Fig. 2 A.

Fig. 2 B.

Fig. 2. Increase in acidity (CO₂ output), sugar disappearance and phosphate distribution during the breakdown of glucose or galactose by an extract prepared from Dutch top yeast grown on galactose. The manometers contained—Main chamber: extract, 0.6 ml.; 0.5 M-NaF, 0.3 ml.; 0.12 M-NaHCO₃, 0.7 ml.; 0.1 M-MgCl₂, 0.1 ml.; 0.25 M-cysteine, 0.1 ml.; glucose (20 mg./ml.), 0.3 ml. in A; galactose (20 mg./ml.), 0.3 ml. in B. Contents made up to 2.1 ml. with distilled water. Side bulb: 0.12 M-NaHCO₃, 0.25 ml.; ATP (2.5 mg. P_{7 min.}/ml.), 0.5 ml. Gas phase: 95 % N₂ + 5 % CO₂; temp. 25°. ----, control; —, glucose or galactose; ○—○, phosphate hydrolyzable in 7 min. by N-HCl at 100°; ●—●, free sugar disappearance; +—+, increase in acidity; △—△, orthophosphate.

The distribution of phosphoric esters in the products of the reaction between adenosinetriphosphate and glucose or galactose

Manometers were set up as described in Fig. 2, and the following quantities were measured during the course of the reaction: (1) increase in acidity, by the CO₂ evolution in bicarbonate buffer, (2) sugar disappearance, (3) change in orthophosphate, and (4) change in acid-labile phosphate.

It can be seen from Fig. 2 that the increase in acidity roughly paralleled the disappearance of either glucose or galactose. With glucose (Fig. 2A) there was a decrease of 0.475 mg. in the amount of acid-labile P, corresponding to an esterification of 2.81 mg. glucose, while actually 3.2 mg. glucose disappeared. Thus 87.5 % of the glucose phosphorylated can be accounted for as acid-stable phosphoric esters, presumably an equilibrium mixture of aldose-6-phosphate and fructose-6-phosphate. In the case of galactose, however, there was no significant decrease in the amount of acid-labile P during a period in which 2.1 mg. of galactose disappeared. This suggests that an acid-labile ester such as galactose-1-phosphate was formed.

The disappearance of acid-labile P in the presence of glucose, less than of the control, corresponds to more than one of the acid-labile phosphate groups of ATP. This is supported by the figures for the disappearance of glucose. Since yeast hexokinase is said to be specific to ATP, and to have no reaction with ADP (Colowick & Kalckar, 1943), this result

suggests the presence in the extract of a myokinase-like enzyme catalyzing the reaction

To confirm the production of an acid-labile phosphoric ester from galactose, an experiment was performed in which the distribution of P among the various phosphoric esters was determined by separation into soluble and insoluble barium salts, and estimation of inorganic P, acid-labile P, and total P in each fraction.

The results are shown in Table 2 Exp. A. When glucose is the substrate, the ATP-P which disappears (decrease in acid-labile P of insoluble barium salts), is largely accounted for in the soluble barium salts as an acid-stable ester (probably a

grown on glucose will not phosphorylate galactose. Berger, Slein, Colowick & Cori (1945-6) have shown that purified yeast hexokinase will not attack galactose.

Isolation of α -galactose-1-phosphate

The reaction mixture contained: 40 ml. ATP solution (approx. 6 mg. $P_{7 \text{ min.}}$ /ml.); 1 g. galactose; 2 ml. *m*-cysteine; 5 ml. 0.1 *M*- MgCl_2 ; 20 ml. 0.5 *M*-NaF; 40 ml. 0.12 *M*- NaHCO_3 ; 40 ml. extract, and water to a total volume of 150 ml. Portions (50 ml.) were placed in each of three Krebs (1933) vessels which were filled with a N_2/CO_2 mixture containing 5% CO_2 and shaken in a bath at 25°. The reaction was allowed to proceed until no more galactose disappeared. Trichloroacetic acid (20 ml. of 40%) was then added to the combined contents of the vessels, and the precipitate centrifuged and

Table 2. *Distribution of phosphoric esters formed by the action of an extract prepared from galactose-adapted Dutch top yeast on a mixture of galactose or glucose and adenosinetriphosphate*

(Exp. A, normal extract; initial acid-labile P=3.62 mg. Exp. B, extract prepared at 0°; initial acid-labile P=3.21 mg. The manometers contained—Main chamber: 0.5 *M*-NaF, 0.3 ml.; 0.1 *M*- MgCl_2 , 0.3 ml.; 0.25 *M*-cysteine, 0.3 ml.; 0.12 *M*- NaHCO_3 , 1.4 ml.; ATP (3.4 mg. $P_{7 \text{ min.}}$ /ml.), 1.0 ml.; and where added, glucose or galactose (20 mg./ml.), 0.8 ml. Contents made up to 4.1 ml. with distilled water. Side bulb: extract, 0.5 ml.; 0.5 *M*-NaF, 0.1 ml.; 0.12 *M*- NaHCO_3 , 0.3 ml. Gas phase: 95% N_2 + 5% CO_2 ; temp. 25°. CO_2 output followed until complete (150 min. in A, 280 min. in B). Contents of manometers then separated into soluble and insoluble barium salts and analyzed.)

	Soluble Ba salts					Insoluble Ba salts				Total P	
	Inorganic P (mg.)	Acid-labile P (mg.)	Acid-stable P (mg.)	Increase in acid-labile P* (mg.)	Increase in acid-stable P* (mg.)	Inorganic P (mg.)	Acid-labile P (mg.)	Acid-stable P (mg.)	Decrease in acid-labile P* (mg.)	Initial (mg.)	Recovered (mg.)
Exp. A											
Control	0.00	0.24	0.48	—	—	0.74	2.86	1.92	—	6.81	6.24
Glucose	0.04	0.42	1.78	0.18	1.30	0.74	1.38	1.68	1.48	6.81	6.04
Galactose	0.02	1.28	0.64	1.04	0.16	0.76	1.80	1.96	1.06	6.81	6.26
Exp. B											
Control	0.00	0.12	0.19	—	—	0.31	2.50	1.56	—	4.82	4.37
Glucose	0.00	0.18	2.29	0.06	2.10	0.28	0.67	1.26	1.83	4.82	4.34
Galactose	0.00	1.13	1.00	1.01	0.81	0.36	0.96	1.16	1.54	4.82	4.25

* Compared with control.

mixture of aldose-6-phosphate and fructose-6-phosphate), and only a small amount of an acid-labile ester is found in this fraction (probably glucose-1-phosphate). When galactose is the substrate, the situation is reversed, and the major part of the ATP-P disappearing is recovered in the soluble fraction as an acid-labile ester. The proportions of acid-labile to acid-stable P in the soluble barium salts are 0.14:0.86 and 0.87:0.13 for glucose and galactose respectively.

The experiment described in Table 2, Exp. A, was repeated using an extract prepared from non-adapted Dutch top yeast. In this case, 96% of the galactose remained unchanged after 150 min. and the CO_2 evolution was 165 μl . compared with 161 μl . in the control. In the same time, 74% of the glucose disappeared and the corresponding CO_2 evolution was 871 μl . Thus it appears that Dutch top yeast

washed with 5% trichloroacetic acid. The supernatant and washings were brought to pH 8.5 with 10% NaOH, and barium acetate (50 ml. of 25%) added. The precipitate was centrifuged and the supernatant poured off and kept. The precipitate was then dissolved in 25 ml. 0.1 *N*-HCl, the pH brought to 8.5 with 10% NaOH and the precipitate centrifuged and discarded. The double extraction was necessary since the first precipitate contains some 'barium-soluble' material in the form of double salts (Cori & Cori, 1932). Two vol. ethanol were added to the combined supernatants, and the mixture left at 0° for 2 hr. The precipitate was then centrifuged, washed in turn with 75% ethanol, 96% ethanol and ether and dried *in vacuo* over CaCl_2 . From 0.57 g. galactose metabolized, 3.18 g. of crude soluble Ba salt were obtained, containing 124.1 mg. P of which 73.4 mg. was acid-labile.

The crude Ba salts contained an impurity of carbohydrate nature, which was removed by taking advantage of the solubility of the K salts of hexosemonophosphates at pH 6.0 in 60% ethanol; under these conditions, polysaccharides are

precipitated. The salt was taken up in 200 ml. water, the insoluble material being discarded, the Ba^{++} removed from the supernatant by addition of a slight excess of saturated K_2SO_4 , and the pH adjusted to 6.0 with 10% KOH; 3 vol. ethanol were then added. The mixture was left at 0° overnight, and the precipitate discarded. The supernatant was concentrated *in vacuo* to 40 ml., and the precipitate which formed again discarded. The supernatant was brought to pH 8.4 with 10% NaOH, and 5 ml. 25% barium acetate and 2 vol. ethanol were added. After leaving at 0° overnight, the precipitate was washed and dried in the usual manner; wt. 1.281 g.; P, 5.1%, of which 88.6% was acid-labile. The recovery of acid-labile P from the crude Ba salt was 79.2%. A sample of the material, after treatment with $Ba(OH)_2$ and $ZnSO_4$, was free from Cu-reducing substance. Another sample was hydrolyzed for 7 min. in N-HCl, and the sugars so produced were subjected to a differential fermentation. Ba salt (50 mg., containing 2.27 mg. acid-labile P) yielded 12.9 mg. of reducing sugar (equivalent to 2.25 mg. P), of which only 8% was fermented by glucose-grown yeast and a further 92% by galactose-grown yeast. It is assumed, therefore, that 8% of the acid-labile P was present as a glucose ester, and 92% as a galactose ester. The salt contained negligible amounts of pentose and fructose, the acid-stable P probably being present largely as aldose-6-phosphate.

Any reducing phosphates were removed by alkaline hydrolysis as follows. The salt (1.136 g.) was dissolved in 100 ml. water and Ba^{++} removed with 5 ml. $N-H_2SO_4$. The precipitate was centrifuged and washed with 5 ml. water. To the supernatant and washings were added 27 ml. N-NaOH and hydrolysis was allowed to proceed at 100° for 30 min. After cooling, the solution was brought to pH 8.4 with 2N-HCl, 25 ml. 25% barium acetate added, and the precipitate collected in the usual manner. Further purification was effected by repeated solution in water and precipitation with ethanol. The final product was identified as barium α -galactose-1-phosphate as follows.

Identification of the product as barium α -galactose-1-phosphate. The salt was dried over P_2O_5 *in vacuo* to a constant weight of 474 mg.; P, 7.49%; acid-labile P, 7.47%. (Calc. for $C_6H_{11}O_9PBa$: P, 7.85%.) The recovery of acid-labile P from the crude barium salt was 48.1%. The product contained less than 0.1% N, no detectable reducing power as measured by the method of Somogyi (1945), and negligible amounts of pentose and ketose. Further evidence on its identity was obtained as follows.

(1) *Sugar liberated on hydrolysis.* Hydrolysis in N-HCl at 100° for 7 min. resulted in the simultaneous liberation of reducing sugar and inorganic P in equimolecular amounts. On hydrolysis and differential fermentation of the sugars liberated, 25 mg. Ba salt yielded 0.6 mg. 'glucose' (2.4%), 10.2 mg. 'galactose' (40.8%), and 1.76 mg. inorganic P, giving a molecular ratio galactose:acid-labile P of 0.99:1.00. (Barium galactose-1-phosphate requires galactose, 45.5%.)

(2) *Specific rotation.* The Ba salt had $[\alpha]_{5461}^{20} = +110^\circ$. This agrees well with the value found by Kosterlitz (1943a) of 109.5° for the Ba salt of synthetic α -galactose-1-phosphate, and with that of 113° for the Ba salt of the natural ester calculated by the same author from the rotation of an impure preparation. Reithel (1945) found the specific rotation of the barium salt of β -galactose-1-phosphate to be $[\alpha]_D^{30} = +34^\circ$.

(3) *Hydrolysis constant.* Hydrolysis was carried out in 0.25N-HCl at 25° using the K salt of the ester, the galactose

liberated being measured by the Somogyi (1945) method and the inorganic P by the Fiske & Subbarow (1925) method.

The hydrolysis constant in 0.25N-HCl at 25° was 0.86×10^{-3} (Table 3). The corresponding value for the K salt of synthetic α -galactose-1-phosphate was found by Kosterlitz (1939) to be 0.89×10^{-3} , and the same author (Kosterlitz, 1943a) calculated the hydrolysis constant for the K salt of the natural ester from the hydrolysis rate of an impure preparation to be 0.91×10^{-3} .

Table 3. *Acid hydrolysis of the acid-labile galactose ester*

(Hydrolysis at 25° in 0.25N-HCl. Initial concentration of acid-labile P = 2.93 mm. Initial concentration of galactose = 2.88 mm. The hydrolysis constant k was calculated from the formula $k = \frac{1}{t_2 - t_1} \log_{10} \frac{a - x_1}{a - x_2}$).

Time (min.)	Galactose liberated (% of total galactose)	$k \times 10^3$	Inorganic P liberated (% of total acid-labile P)	$k \times 10^3$
100	18.2	0.87	18.3	0.88
200	32.7	0.85	32.9	0.86

On the basis of this evidence it is concluded that the easily hydrolyzable ester formed from galactose and ATP is α -galactose-1-phosphate.

Possible formation of galactose-6-phosphate as an intermediate

To test the possibility that the primary phosphorylated product in galactose fermentation is galactose-6-phosphate, which is immediately transformed to galactose-1-phosphate, the effect of adding synthetic galactose-6-phosphate to the enzyme system phosphorylating galactose was studied. The results are shown in Table 4. In the presence of galactose-6-phosphate, the distribution of phosphoric esters did not differ significantly from that of the control, and there was no diminution in the amount of acid-stable P during the course of the reaction. These facts, together with the absence of increased acid formation (CO_2 evolution) over the control, indicate that galactose-6-phosphate is not attacked in a system which is able to form an easily hydrolyzable phosphoric ester from galactose. Galactose-6-phosphate is, therefore, not an intermediate in the formation of galactose-1-phosphate in this system.

The action of the enzyme system on D-tagatose, the ketose corresponding to D-galactose, was also investigated. Tagatose was not fermented by intact cells of galactose-adapted yeast, but this does not necessarily exclude it from playing a part in galactose fermentation analogous to that of fructose in glucose fermentation. It can be seen, however, from Table 4, that tagatose was not phosphorylated by an extract of galactose-adapted cells. The figures for the P distribution in the presence of tagatose were almost identical with those in the control.

Table 4. *Distribution of phosphoric esters formed by the action of an extract prepared from galactose-adapted Dutch top yeast on a mixture of galactose, galactose-6-phosphate or tagatose, and adenosinetriphosphate*

(The manometers contained—Main chamber: 0.5M-NaF, 0.3 ml.; 0.1M-MgCl₂, 0.3 ml.; 0.25M-cysteine, 0.3 ml.; 0.12M-NaHCO₃, 1.4 ml.; ATP (4.0 mg. P_{7 min}/ml.), 1.0 ml.; and, where added, galactose or tagatose (20 mg./ml.) or galactose-6-phosphate (1.88 mg. P/ml.), 0.8 ml. Side bulb: extract, 0.5 ml.; 0.5M-NaF, 0.1 ml.; 0.12M-NaHCO₃, 0.2 ml. Gas phase: 95% N₂+5% CO₂; temp. 25°. CO₂ output was followed until complete (210 min.), and the phosphate esters formed were then separated into soluble and insoluble barium salts and analyzed.)

	Initial P		Soluble Ba salts				Insoluble Ba salts			
	Control: galactose or tagatose (mg. P)	Galactose-6-phosphate (mg. P)	Control (mg. P)	Galactose (mg. P)	Galactose-6-phosphate (mg. P)	Tagatose (mg. P)	Control (mg. P)	Galactose (mg. P)	Galactose-6-phosphate (mg. P)	Tagatose (mg. P)
Ortho-P	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.24	0.23	0.21
Acid-labile P	3.92	3.92	0.14	1.52	0.16	0.14	3.19	1.60	3.23	3.07
Change in acid-labile P*	—	—	—	+1.38	+0.02	0.00	—	-1.59	+0.04	-0.12
Acid-stable P	2.15	3.65	0.25	0.29	1.70	0.25	1.45	1.37	1.55	1.50
Change in acid-stable P*	—	+1.50	—	+0.04	+1.45	0.00	—	-0.08	+0.10	+0.05
CO ₂ in 210 min. (μl.)	—	—	210	726	214	221	—	—	—	—

* Compared with control.

The production of acid-stable phosphoric esters from galactose

It was found that if precautions were taken to keep the temperature down to 0° during the breakdown of the yeast cells and the purification of the enzyme, there was occasionally obtained from galactose an acid-stable ester which appears in the soluble barium salts. Typical results for the distribution of P with such an extract are given in Table 2, Exp. B. While the phosphorylation of glucose results in the formation of an acid-stable ester (2.10 mg. P), that of galactose gave a mixture of an acid-labile ester (1.01 mg. P) and an acid-stable ester (0.81 mg. P). It seems that if these precautions about cooling are taken, a second adaptive enzyme, which is destroyed during the more drastic procedure, is present and forms an acid-stable ester from galactose-1-phosphate.

Using an extract prepared with particular attention to adequate cooling, an attempt was made to isolate the acid-stable ester formed from galactose. The preparation was set up in the same way as for the isolation of α -galactose-1-phosphate described above, but using half the quantities. After 4 hr., 210 mg. of galactose had disappeared.

The soluble Ba salts were isolated as described previously: wt. 907 mg.; P, 5.9%, of which 24.9% was acid-labile and 75.1% acid-stable. Differential fermentation of the sugar produced by hydrolysis in N-HCl at 100° for 7 min. showed it to consist of 86% 'galactose' and 14% 'glucose', and, therefore, the acid-labile ester was probably chiefly galactose-1-phosphate. The Ba salt (500 mg. $\equiv$ 22.2 mg. acid-stable P) was converted into the Na salt (120 mg.), and 15 ml. 5N-HCl added. The acidified solution was boiled for 7 min. to hydrolyze the acid-labile phosphoric esters present, cooled to room

temp., the pH brought back to 8.4 with 10% NaOH, and 3 ml. 25% barium acetate added. The precipitate was centrifuged, washed with 10 ml. water, and 2 vol. 96% ethanol were added to the combined supernatants. The precipitate was centrifuged, washed with ethanol and ether, and dried over CaCl₂ *in vacuo*. The salt was redissolved in 25 ml. water, the insoluble material being spun off, reprecipitated with 2 vol. ethanol, washed with ethanol and ether, and dried over CaCl₂ *in vacuo*; wt. 212 mg.; P, 6.18%; acid-labile P, nil. (Recovery of acid-stable P = 59%.)

The pentose and ketose in the precipitate were negligible, and the total N was less than 0.1%. The reducing value of the salt, estimated by a Somogyi copper reagent standardized against glucose-6-phosphate, was equivalent to a content of 59.5% barium glucose-6-phosphate (corresponding to 78.2% of the total acid-stable P). No insoluble methylphenylhydrazone could be obtained from the barium-free esters, showing that galactose-6-phosphate was not a constituent (Grant, 1935).

The ester was hydrolyzed by a non-specific phosphatase prepared from a dialyzed extract of homogenized rat intestine. A solution of the Na salt (10 ml. containing 3.24 mg. P) was incubated for 2 hr. at 37° with 5 ml. extract, 25 ml. acetate buffer (pH 5.5), and 1 ml. 0.1M-MgCl₂; the inorganic P liberated corresponded to 89.5% of the total P and the P:sugar ratio was 1.00:0.705. For comparison, a similar mixture was set up using sodium glucose-6-phosphate (prepared from 50 mg. anhydrous barium glucose-6-phosphate) in place of the unknown Na salt; the inorganic phosphate liberated was 84% of the total, and the P:sugar ratio was 0.91:1.00.

These figures, together with those given in the previous paragraph, suggest that the unknown salt was a mixture of acid-stable esters, one of which is non-reducing and produces a non-reducing substance on hydrolysis, and the other is reducing and contains a reducing sugar. It has not yet been possible to

investigate these esters more completely, but it seems reasonable to suppose that the reducing ester is an aldose-6-phosphate, probably glucose-6-phosphate. The non-reducing ester may be trehalose-monophosphate (Robison & Morgan, 1930; Grant, 1935).

The formation of galactose-1-phosphate during adaptation to galactose fermentation

Reiner (1947) investigated the change in acid-labile P during the period immediately preceding the onset of adaptation to galactose fermentation by *Saccharomyces cerevisiae*, and concluded that fermentation was preceded by the accumulation of ATP. He did not separate the phosphoric esters into soluble and insoluble barium salts, however, and in view of the findings presented in this paper, his results might equally well have been explained by the accumulation of galactose-1-phosphate in the prefermentative period, thus stimulating the formation of a second adaptive enzyme attacking galactose-1-phosphate with the production of an acid-stable phosphoric ester. This point was investigated by the author using a separation into soluble and insoluble barium salts to differentiate between ATP and galactose-1-phosphate.

Fig. 3. Distribution of phosphoric esters during the adaptation of Dutch top yeast to the fermentation of galactose. O—O, phosphorus hydrolyzable in 7 min. in the insoluble barium salt fraction; ●—●, orthophosphate; +—+, phosphorus hydrolyzable in 100 min.—alkali-labile phosphorus in the soluble barium salt fraction; △—△, respiratory quotient.

The separation was carried out according to the method of Umbreit *et al.* (1945), and inorganic phosphate was estimated by the method of Berenblum & Chain (1938). The remainder of the experiment was carried out as described by Reiner (1947), but using Dutch top yeast and incubating at 25°. The results are shown in Fig. 3. It can be seen that, in the period immediately preceding adaptation

(fermentation), there was no accumulation of P in the fraction ($P_{100} - P_{\text{alkali labile}}$) which would have contained galactose-1-phosphate.

DISCUSSION

From the work described in this paper, it seems reasonably certain that the first step in galactose fermentation by Dutch top yeast is the transference of the terminal phosphate group from ATP to galactose to give galactose-1-phosphate. The enzyme catalyzing this reaction is activated by Mg^{++} and by cysteine, and clearly belongs to the class of phosphokinases. It is proposed to call it galactokinase.

By analogy with hexokinase it might be expected that galactose-6-phosphate would be the primary product, but this seems very unlikely for the following reasons: (1) no yeast extract yet investigated can ferment galactose-6-phosphate (Hvistendahl, 1932; Grant, 1935); (2) the enzyme system which forms galactose-1-phosphate from galactose is unable to attack galactose-6-phosphate (Table 4); (3) there is no initial formation of an acid-stable ester during the phosphorylation of galactose by the extract (Fig. 2); (4) the extract is unable to phosphorylate tagatose, whereas yeast hexokinase will phosphorylate fructose and mannose in addition to glucose.

It has been shown by Spiegelman, Reiner & Morgan (1947) that the acquisition of the ability to ferment galactose by *Saccharomyces cerevisiae* involves the formation of one or more apoenzymes. The work described in this present paper shows that one of these enzymes is galactokinase.

Since galactose is fermented with the formation of fructose-1:6-diphosphate (Grant, 1935), the question arises as to how the galactose-1-phosphate is transformed into hexosediphosphate. The answer to this question is still obscure, but some indication is given by the work of Kosterlitz (1943b), and by the experiments described in this paper in which a proportion of the phosphate esterified was present as an acid-stable phosphoric ester with a soluble barium salt. If, as seems probable, this proves to be glucose-6-phosphate, then it seems reasonable to suppose that the transformation of galactose-1-phosphate to fructose-1:6-phosphate takes place via glucose-6-phosphate or an ester in equilibrium with glucose-6-phosphate in the system described.

SUMMARY

1. Dutch top yeast has been shown to produce adaptively an enzyme, named galactokinase, which catalyzes the phosphorylation of galactose by adenosinetriphosphate forming an ester which has been identified as α -galactose-1-phosphate. The enzyme requires Mg^{++} and cysteine for full activity.

2. Galactose-6-phosphate is not an intermediate in this reaction.

3. The formation of an acid-stable ester from galactose has been observed occasionally with extracts of galactose-adapted yeast.

4. There is no evidence for the accumulation of galactose-1-phosphate during the pre-adaptive period.

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Studies on the Lens

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1. POST-MORTEM CHANGES IN THE MINERAL CONTENT

Our knowledge of the composition and metabolism of the lens has been gained almost exclusively from work on the excised organ, and it is therefore of first importance that post-mortem changes should be fully understood. It might be thought that the use of normal aqueous humour as a bathing medium for the excised lens would make such a study supererogatory, but the results described below show that, even when the lens is left undisturbed in the dead eye, important changes in its mineral content (characteristic of those taking place in a dying tissue) occur within a few hours.

EXPERIMENTAL

To minimize variance due to age, adult rabbits were used in all the experiments described with this species. Except where otherwise stated, the sheep eyes were received from the slaughterhouse within approximately 2 hr. of death.

After removing the aqueous humour and cutting away the posterior half of the eye the lens was removed by placing the anterior half of the eye on a flat dish, cornea downwards, reflecting back the curved scleral wall and cutting the suspensory ligament. Before being weighed, the lens was blotted on filter paper. For analysis, the proteins were precipitated by the Somogyi (1930) method and the filtrate made up to a definite volume. Chloride was estimated by the AgIO_3 method of Sendroy (1937). When radioactive isotopes were used, activity was measured in the filtrate on a standard Geiger counter apparatus with the counter described by Maurice (1948). Radioactive sodium (^{24}Na) in the form of NaCl , and bromine (^{82}Br) in the form of NH_4Br were injected intravenously in isotonic solutions, without anaesthesia. After the experimental period the animal was killed by a blow on the neck, and the eyes, together with blood samples, were taken immediately.

For the *in vitro* study of the 'diffusion out' of chloride from the lens, the latter was placed in isotonic Na_2SO_4 solution, while for the 'diffusion in' of sucrose, aqueous humour was diluted with 0.3 vol. of an isotonic sucrose solution. Sucrose was determined by hydrolyzing the Somogyi filtrate of the macerated lens with dilute H_2SO_4 , and estimating the reducing value by the Hagedorn-Jensen method.