and pK'_2 by means of Henderson-Hasselbalch's equation

$$pK'_a = p\mathrm{H} - \log \frac{B}{C-B}$$

[Van Slyke, 1922]. In these equations B stands for molar concentration of total alkali added and C for molar concentration of galactose-1-phosphate in the titration mixture at any given time.

The values found for orthophosphoric acid, pK'_1 1·95 and pK'_2 6·83, agree well with those recorded in the literature [Van Slyke, 1922; Meyerhof & Suranyi, 1926; Cori *et al.*, 1937]. Galactose-1-phosphoric acid has pK'_1 1·00 and pK'_2 6·17 while hexose-6-phosphoric acid (Robison ester) has pK'_1 0·94 and pK'_2 6·11, fructose-6-phosphoric acid pK'_1 0·97 and pK'_2 6·11 [Meyerhof & Lohmann, 1927] and glucose-1-phosphoric acid pK'_1 1·10 and pK'_2 6·13 [Cori *et al.*, 1937]. There is no significant difference between the biologically important pK'_2 values of galactose-1-phosphoric acid and those of the other hexosemonophosphates.

Since Meyerhof & Lohmann [1927] found that the pK_2 values of fructose-6-phosphoric and hexose-6-phosphoric (Robison) acids were decreased by about 0·14 by the addition of 0·1 *M* neutral salt solution, 10 ml. 0·1 *M* K_2 galactose-1-phosphate solution were titrated with *N* HCl. pK'_2 determined in this way was 6·06, as compared with 6·17, the value obtained by titrating the ester with NaOH.

SUMMARY

α(?)-Galactose-1-phosphoric acid, like other hexose-monophosphates, is a stronger acid than is orthophosphoric acid. α(?)-Galactose-1-phosphoric acid has pK'_1 1·00 and pK'_2 6·17, while orthophosphoric acid has pK'_1 1·95 and pK'_2 6·83.

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The Fermentation of Galactose and Galactose-1-phosphate

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Galactose-1-phosphate is present in the livers of rabbits and rats during galactose assimilation [Kosterlitz, 1937; Kosterlitz & Ritchie, 1943*b*]. No information as to the fate of this ester has so far been obtained except that it is more resistant to the phosphatase of liver than is glucose-1-phosphate [Cori, Colowick & Cori, 1938; Kosterlitz & Ritchie, 1941]. Since the carbohydrate metabolism of yeast cells is strikingly similar to that of mammalian tissue, it was decided to examine the fermentation of galactose and galactose-1-phosphate and compare the results with those obtained in the fermentation of glucose and glucose-1-phosphate.

The ability of some yeasts to ferment galactose is due to the formation of 'galactozymase' during adaptation. This process takes place in aqueous solutions of galactose and can occur without cell multiplication [Stephenson & Yudkin, 1936; for the older literature see that paper, and Harden, 1932]. Yeast juice [Harden & Norris, 1910; Grant, 1935], dried yeast and maceration extract [Abderhalden, 1925] prepared from adapted yeast, will ferment galactose. In the products of fermentation Nilsson [1930] found, besides Harden-Young ester, a monophosphate with a specific rotation higher than that of Robison ester. No data as to the reducing power of this ester and the nature of its constituent hexose were given. Grant [1935] confirmed the presence of 1:6-fructose diphosphate, and found trehalose-monophosphate and Robison ester. Using the methylphenylhydrazine test, he could not find any evidence for the accumulation of a galactose phosphoric ester. He further could demonstrate that galactose-6-phosphate was not fermented by galactose-adapted yeast.

EXPERIMENTAL

Materials and methods

Maceration extracts. These were prepared in the usual manner from a pure strain of *Saccharomyces cerevisiae* (*S. cerevisiae*) Frohberg, which had been propagated in this laboratory since 1935, when it was generously supplied by

Dr H. B. Hutchinson of the Distillers Company, Ltd. It has been subcultured in broth containing 0·8% dried 'Difco' yeast extract and 3% galactose. For the preparation of dried yeast, the yeast was grown in aerated broth in lots of 1½–3 l. at 25° for 48 hr.

Galactose. In order to remove glucose, a 10% solution of commercial galactose was incubated with 2% *Saccharomyces ludwigii* (*S. ludwigii*) at 28° for 48 hr.; after centrifuging off the yeast and concentrating the solution under reduced pressure, the galactose was crystallized several times from 70% ethanol. It had $[\alpha]_{5461}^{17°} + 95·0°$.

Glucose-1-phosphate. The K_2-salt was prepared according to Hanes [1940].

Galactose-1-phosphate. The K_2-salt was prepared by the method given in a previous paper [Kosterlitz, 1939*a*]. Since the K_2-salts of the esters are alkaline, HCl was added to obtain the desired *p*H. The quantities of acid required were read from electrometric titration curves [Kosterlitz, 1943].

CO_2 production. This was followed in Warburg manometers shaken in a bath at 25°. The gaseous phase was N_2; any O_2 present in the commercial nitrogen was absorbed by passing through two gas-washing bottles containing a solution of 16 g. $Na_2S_2O_4 . 2H_2O$ and 2 g. Na-anthraquinone-2-sulphonate in 100 ml. 13% NaOH [Fieser, 1924] and through a third bottle containing 13% NaOH.

Estimation of glucose-1-phosphate and galactose-1-phosphate. The esters were determined in trichloroacetic acid filtrates by the method described by Kosterlitz & Ritchie [1943*a*].

RESULTS

The results of three typical experiments are given in Fig. 1*a–c*. In all experiments, whether with hexoses or esters, typical curves of CO_2 production were obtained, showing an induction period, a period of rapid fermentation (period of esterification), and finally, a period of slow fermentation (period of hexosediphosphate fermentation). Meyerhof & Lohmann [1927] found that only the naturally occurring hexosemonophosphates, namely, Neuberg and Robison esters, gave such curves, while synthetic esters which were not intermediaries in alcoholic fermentation produced CO_2 at a slow and uniform rate.

In the first experiment (Fig. 1*a*), notwithstanding the addition of a small quantity of hexosediphosphate to the fermentation mixture, the CO_2 curves of the free hexoses showed a fairly long induction period and an autocatalytic shape. The maximum rates of CO_2 production were 80 μl./5 min. with glucose, and 62 μl./5 min. with galactose. On the other hand, the curves obtained with glucose-1-phosphate and galactose-1-phosphate were practically identical. Their maximum rates were 45–50 μl. CO_2/5 min. It is to be noted that, since in these experiments inorganic P was present in excess, the shape of the CO_2 curves was wholly conditioned by the substrate. Thus, during the rapid period of fermentation, glucose produced 575 μl. CO_2 and galactose 500 μl. CO_2 instead of the expected 1090 μl., or about 50%, while the esters produced 300 μl. CO_2 or 27·5% of the theoretical value. This means that while the hexoses obeyed the Harden-Young equation the esters did not. These results are in good agreement with those of Meyerhof & Lohmann [1927] who, investigating the action of maceration extract on Neuberg and Robison esters, found a CO_2 production of 25% of the calculated value, and with those of Harden & Robison [Harden, 1932] who found that during fermentation with zymin, only 20–25% of the added Robison ester was fermented at the enhanced rate. The lower CO_2 production by the monophosphates can be explained by the facts that, compared with the hexoses, they can accept only one-half the quantity of phosphate and that, according to Warburg & Christian [1939], the partial dephosphorylation of 1:3-diphosphoglyceric acid to 3-phosphoglyceric acid by the hexokinase-adenosine diphosphate-hexose system is a limiting factor in alcoholic fermentation by maceration extract.

Addition of catalytic quantities of acetaldehyde and hexosediphosphate (Fig. 1*b*) almost abolished the induction period in the fermentation of the free hexoses, with a simultaneous increase of the maximum rates of CO_2 production. In the case of glucose the maximum rate rose from 80 to 160 μl./5 min., and in the case of galactose from 62 to 75 μl./5 min. Although the addition of acetaldehyde caused a marked difference between the fermentation rates of free glucose and free galactose, it scarcely influenced the fermentation of either of the 1-esters.

When acetaldehyde alone was added to the fermentation mixture, the difference between the fermentations of glucose and galactose became rather more marked than in the experiment with addition of acetaldehyde and hexosediphosphate. There was a slight difference between the rates of CO_2 production in the case of the 1-esters in favour of glucose-1-phosphate (Fig. 1*c*).

If the yeast was dried slowly in the manner suggested by Lipmann [1938], the maceration extract obtained from it was four times as active as were extracts from yeasts dried in the ordinary way. In the presence of acetaldehyde, the maximum rates of CO_2 evolution/5 min. were as follows: glucose 690 μl., galactose 270 μl., glucose-1-phosphate and galactose-1-phosphate 200 μl. With glucose-1-phosphate the maximum fermentation rate was attained sooner than with galactose-1-phosphate, otherwise there was no difference between the fermentations of the two esters.

The experiments described so far indicate that galactose-1-phosphate plays the same role in galactose fermentation as do glucose-1-phosphate and glucose-6-phosphate in glucose fermentation. Grant [1935] found that Robison ester and 1:6-fructose diphosphate accumulate during fermentation of galactose by adapted yeast preparations, and that

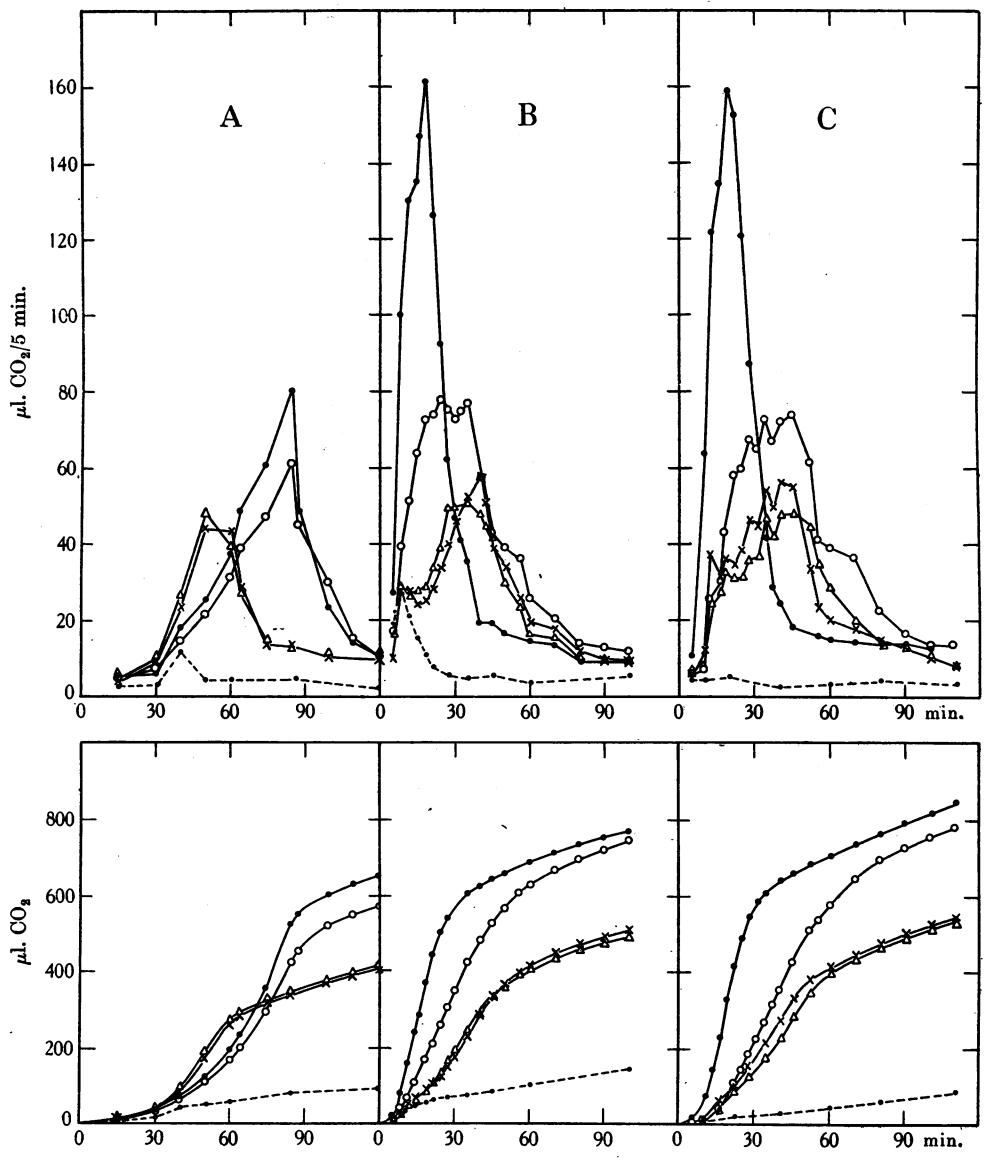

Fig. 1. Maceration extract from *Saccharomyces cerevisiae* Frohberg grown on galactose broth. 1 part dried yeast and 4 parts water were incubated at 35° for 2 hr. The extract was obtained by centrifuging at 4000 r.p.m. 1 ml. contained 2·55 mg. inorganic P; *p*H 5·8.

Main chamber: 1 ml. extract.

Side-bulb: (*a*) 0·05 ml. K_2-hexosediphosphate (0·115 mg. P), and either 0·25 ml. hexose solution (=4·37 mg. hexose=1090 μl. CO_2) in 0·097 *M* phosphate buffer of *p*H 6·5, or 0·25 ml. ester solution (=4·37 mg. hexose) in 0·0267 *N* HCl (*p*H 6·5).

(*b*) 0·05 ml. containing Mg-hexosediphosphate (0·07 mg. P) and 1 mg. acetaldehyde, and either 0·2 ml. hexose solution (=4·5 mg. hexose=1120 μl. CO_2) in 0·12 *M* phosphate buffer of *p*H 6·5, or 0·2 ml. ester solution (=4·5 mg. hexose) in 0·0338 *N* HCl (*p*H 6·5).

(*c*) as in (*b*), but hexosediphosphate was omitted.

····· Autofermentation; •—• glucose; o—o galactose; ×—× glucose-1-phosphate; Δ—Δ galactose-1-phosphate.

galactose-6-phosphate is not an intermediary in galactose fermentation. The question, therefore, arises of how galactose-1-phosphate is converted to Robison ester: Is galactose-1-phosphate transformed to glucose-1-phosphate, which in turn is acted upon by phosphoglucomutase and isomerase to form Robison ester? Preliminary experiments with dialysed maceration extracts were not conclusive on account of the presence of phosphoglucomutase in such extracts. To give an example: 0·75 ml. extract, dialysed for 2 hr. + 0·5 ml. 0·08 *M* veronal acetate buffer of *p*H 6 + 0·25 ml. 0·06 *M* glucose-1-phosphate or galactose-1-phosphate adjusted to *p*H 6 were incubated at 25° for 30 min. In the case of glucose-1-phosphate 83 % of the ester was converted to difficultly hydrolysable ester (Robison), 14 % was accounted for by inorganic P, while 3 % remained unaltered. In the case of galactose-1-phosphate only 27 % was converted to difficultly hydrolysable ester, 11 % was accounted for by inorganic P, while 62 % remained unaltered. The structure of the difficultly hydrolysable ester formed from galactose-1-phosphate has not yet been identified but it is likely to be Robison ester, since galactose-6-phosphate can be ruled out. No glucose-1-phosphate was present in the reaction mixture of the experiment with galactose-1-phosphate, a finding which had to be expected in view of the presence of highly active phosphoglucomutase. Unfortunately, phosphoglucomutase cannot be inhibited specifically. Attempts to reduce the activity of phosphoglucomutase by prolonged dialysis were accompanied by great losses in the activity of the galactose-1-phosphate-converting enzyme; the activity could not be restored by Mg^{++} ions which, in extracts dialysed for 2 hr., accelerated the disappearance of galactose-1-phosphate. Fractionation with $(NH_4)_2SO_4$ has so far been unsuccessful; both the galactose-1-phosphate-converting enzyme and phosphoglucomutase were absent from the proteins precipitated by either 50 or 70 % saturation.

Notwithstanding the great differences between the fermentation rates of the non-phosphorylated hexoses, the fermentation curves of the 1-esters are so similar to each other as to suggest the existence of an enzymatic equilibrium between the esters. However, in order to obtain further information on the mechanism which causes the conversion of galactose-1-phosphate to Robison ester, the attempts to isolate the enzyme, or at least to separate it from phosphoglucomutase, will have to be taken up again on a scale larger than is possible at present.

From the facts described in this paper, a tentative hypothesis of galactose fermentation may be formulated as follows: During the adaptation of *S. cerevisiae* Frohberg to ferment galactose at least two new enzymes are formed. Enzyme (1) phosphorylates galactose at C_1, probably by a system similar to the hexokinase-adenylpyrophosphate systems which phosphorylate glucose and fructose at C_6 and not at C_1 [Colowick & Kalckar, 1941]. Enzyme (2) converts galactose-1-phosphate to Robison ester, probably by way of glucose-1-phosphate, which in turn is acted upon by phosphoglucomutase or isomerase. The fermentation rate of galactose is lower than that of glucose; the rates of reaction (1) or (2) or of both constitute the limiting factors. During the process of adaptation, yeast may be fully adapted to ferment galactose-1-phosphate and, at the same time, be incompletely adapted to ferment non-phosphorylated galactose; when this is the case, galactose will be fermented more slowly than the 1-phosphate. This phenomenon, which was observed in two out of five samples of dried yeast [Kosterlitz, 1939*b*; 1942], supports the view that two new enzymes are formed during adaptation. Non-adapted yeast ferments neither galactose nor galactose-1-phosphate.

SUMMARY

1. With maceration extract, both glucose-1-phosphate and galactose-1-phosphate showed the characteristics of natural hexosemonophosphates, namely, the three periods of induction, rapid fermentation, and slow fermentation. While the non-phosphorylated hexoses produced about 50 % of the calculated quantity of CO_2 during the period of rapid fermentation, the esters produced only 25–30 % during the same period. The fermentation rates of the esters were so similar as to suggest an enzymatic equilibrium between them. So far it has not been possible to supply direct experimental proof for this suggestion.

2. A tentative hypothesis of galactose fermentation is formulated: it is assumed that during the adaptation of *Saccharomyces cerevisiae* Frohberg to ferment galactose, two new enzymes are formed, one which phosphorylates galactose at C_1 and another which converts galactose-1-phosphate to Robison ester, probably by way of glucose-1-phosphate.

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The Oxidation of Ascorbic Acid by *o*-Dinitrobenzene, and the Detection of Dehydroascorbic Acid

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During a survey of the natural sources of ascorbic acid, three reactions have been observed that may be of analytical value: (1) the rapid reduction of *o*-dinitrobenzene by ascorbic acid to a substance giving a violet pigment in alkaline solution, (2) the formation of a green pigment when dehydroascorbic acid is boiled alone in aqueous solution at *p*H 3·5–4·5, and (3) the spontaneous formation of a red pigment when dehydroascorbic acid is kept in alkaline solutions out of contact with air.

(1) *Reaction of ascorbic acid with* o*-dinitrobenzene*

(*a*) *Experimental.* When 2–5 ml. of a dilute (0·02 %) aqueous solution of ascorbic acid are treated in the cold with 5 drops of a saturated aqueous solution of *o*-dinitrobenzene, and then made alkaline by addition of 5 drops 20 % NaOH, a violet colour rapidly develops, and is stable for several hours. Under these conditions the colour is not given by dehydroascorbic acid, cysteine, glutathione, uric acid or creatinine. Fructose gives a slow positive reaction in the cold, but only after 10–15 min., when the solution contains at least 0·2 % of this sugar. The other reducing sugars may require 2 hr. or longer. Proteins, simple alcohols, aldehydes, ketones, and the commoner hydroxy-, keto-, and amino-acids do not give the test. The test loses its selectivity if the reaction mixture is heated, and a positive result is then given by all the reducing sugars and by uric acid. Thus, by controlling temperature and concentration of alkali, it becomes possible to detect ascorbic acid in presence of other compounds of biological importance. On acidification, the violet pigment changes to yellow, and can be extracted by chloroform or by peroxide-free ether. Addition of alkali removes the pigment from the organic solvent, and regenerates the violet colour. By this means, small amounts of ascorbic acid can be detected in solutions the colour of which obscures the test.

(*b*) *History of the reagent.* Lipschitz [1920] reported that living tissues, such as muscle and yeast, contain a catalytic system capable of reducing '*m*-dinitrobenzene' to a yellow pigment, at *p*H 7·4, under anaerobic conditions. Addition of alkali converted the yellow into violet. The test was developed colorimetrically, and it was later found to depend on the presence of *o*-dinitrobenzene as a contaminant in the *m*-isomer [Lipschitz & Osterroth, 1924]. Apparently unaware of this, Ekkert [1934] claimed that reducing sugars gave a violet colour when heated in alkaline solution with *m*-dinitrobenzene, but v. Szecseny-Nagy [1935] showed that this reaction was due also to contamination by the *o*-isomer, and that pure *m*-dinitrobenzene gave no colour. This was confirmed by Truhaut [1937].

(*c*) *Mechanism of the test.* From an inspection of the formulae of the reactants, the test seems to depend on the presence of the enediol system, —C(OH)=C(OH)—, which occurs in ascorbic acid, though, of course, the reagent is not specific for this system, since powerful inorganic reducing agents such as hydrosulphite give a similar response.

Aldoses and ketoses react only when conditic bring about enolization. Kept in alkaline soluti at room temperature for some hours they give immediate violet on addition of the reagent, whic