The Effect of Temperature on the Fermentation of *d*-Mannose by Yeast

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Of all the hexoses found in nature or prepared in the laboratory only *d*-glucose, *d*-fructose and *d*-mannose are fermented by yeasts. On solution in water the crystalline forms of these hexoses exhibit mutarotation resulting in an equilibrium mixture of various tautomeric modifications. Since within the physiological range of temperature this reversible interconversion proceeds quickly, no conclusion can be drawn from experiments of the usual type as to which of the various isomerides present actually undergoes fermentation. In order to obtain more detailed information in this respect fermentations have been carried out at 0°, at which temperature the rate of mutarotation of the hexose added in the solid form is greatly reduced. Under these conditions α -*d*-glucopyranose is found to be freely fermented (Gottschalk, 1943*a*); the same holds for β -*d*-glucopyranose (unpublished). If β -*d*-fructopyranose is added to a yeast suspension at 0°, the rate of fermentation is only a small fraction of that obtained with α -*d*-glucopyranose, and is independent of yeast concentration. In fact the rate is almost identical with that at which the β -*d*-fructopyranose mutarotates into *d*-fructofuranose (Gottschalk, 1943*a*). When yeast at 0° acts upon the equilibrium mixture of *d*-fructose containing the pyranose and furanose forms, a limited amount of the keto-sugar is consumed (Gottschalk, 1943*b*). *d*-Fructofuranose, set free from sucrose or raffinose by saccharase, is readily fermented at 0° (Gottschalk, 1945). From these results it is concluded that only the furanose form of *d*-fructose is suitable for fermentation, thus substantiating a hypothesis first advanced by Hopkins (1931). In the present paper the effect of temperature, from 0 to 25°, on the fermentation of α - and β -*d*-mannopyranose by living yeast and yeast extract is investigated.

MATERIALS AND METHODS

Materials. The experiments were carried out with baker's yeast (Effront), brewer's mild ale top yeast (*Sacch. cerevisiae*) and with brewer's bottom yeast (*Sacch. carlsbergensis*). The brewer's yeast was collected at the Carlton Brewery, Melbourne, and treated as described previously (Gottschalk, 1946). Brewer's bottom yeast was washed and pressed by means of a hand press, the yeast was then dried for 48 hr. either at 27–28° or at 30°. To prepare the Lebedev extract, 20 g. of the dried and thoroughly ground yeast were

well mixed with 60 ml. of boiled yeast juice freshly prepared from the bottom yeast in the usual manner (cf. Neuberg & Gottschalk, 1925). After the addition of 100 mg. *d*-glucose the mixture, contained in a round-bottom flask, was immersed in a water-bath at 25° for 4 hr. or at 35° for 2 hr.; every 10 min. the flask was shaken. Following the extraction, the suspension was cooled and centrifuged. The supernatant liquid was immediately used for the experiment.

The acid-soluble inorganic phosphate concentration of the yeast extract thus prepared is on an average 0.040M, i.e. about the same as that of the intact cell (cf. Gottschalk, 1946). The inorganic phosphate determinations were carried out by the method of Kuttner & Cohen (1927).

The α -*d*-glucopyranose and β -*d*-mannopyranose used in the experiments were pure, anhydrous substances; α -*d*-mannopyranose was prepared from β -*d*-mannopyranose by the method of Levene (1924). The specific rotations of the hexoses (in water; $c=3$, $l=2$) are as follows:

α -*d*-glucopyranose $[\alpha]_D^{20}$: initial value (extrapolated), +108.5°; after 28 hr., +52.4°.

α -*d*-mannopyranose $[\alpha]_D^{20}$: initial value (extrapolated), +26.5°; after 24 hr., +14.4°.

β -*d*-mannopyranose $[\alpha]_D^{20}$: initial value (extrapolated), -16.0°; after 24 hr., +14.2°.

Technique. The experiments were performed at 0° in a refrigerated laboratory (Gottschalk, 1945). Fresh baker's yeast (12 g.) was suspended in 17.6 ml. of 0.1 M-KH₂PO₄, cooled to 0° and equilibrated with nitrogen (freed from oxygen). Immediately before its addition to the suspension the sugar, contained in a small vessel, was dissolved in 0.5 ml. of ice water and the vessel rinsed once with 0.5 ml. of ice water. If an equilibrium mixture of the hexose was to be used in the experiment, the sugar solution was incubated at 25° for 24 hr. and cooled to 0° several hours before the experiment. A corresponding assay without sugar served as control. At the end of the experiment the yeast was spun down at 0° and the supernatant liquid analyzed in triplicate for unfermented sugar by the Hagedorn-Jensen method, using a microburette. It was confirmed that the reducing powers of *d*-glucose and of *d*-mannose are equal (cf. Sobotka & Reiner, 1930).

In the experiments performed at 10 and 25° the rate of fermentation was determined with the apparatus described by Gottschalk & Rawlinson (1942). Constancy of temperature at 10° (± 0.25) was obtained by regulating the room temperature to 10–11° and making the final adjustment inside the thermostat with trays of shaven ice and solid CO₂.

When living cells were used, 1–6 g. of yeast, depending on the temperature of the experiment, were suspended in 0.05 M-KH₂PO₄ (total vol. 50.0 ml.); 20.0 ml. of this suspension (pH 4.5) were pipetted into the bulb of the apparatus. The liquid phase was equilibrated against nitrogen.

The sugar, dissolved either freshly or 24 hr. previously in 1.0–1.4 ml. water, was then added. Readings were taken at 5 min. intervals and the volumes obtained reduced to N.T.P. The highest rate of CO₂ production (ml. CO₂/5 min.) during the first 35 min. of each experiment was taken as the fermentation rate for the given substrate concentration.

In experiments with cell-free preparations 10.0 ml. of the yeast extract (pH = 5.96) plus 0.25 ml. toluene were used in each assay. After equilibration of the system the sugar solution and 0.30 ml. of 1% acetaldehyde were added and readings taken every 4 min. There was no CO₂ production in the control without sugar.

The dry weight of the yeast was determined as before (Gottschalk, 1943*a*). In calculating the sugar concentration inside the living cell it was assumed that 96% of the total water of the cell is available as solvent to an added solute (cf. Hill, 1930). The volume of the yeast cells, as ascertained by haematocrit, did not change appreciably during the experiment. The pH measurements were made with the glass electrode.

RESULTS

It may be seen from Table 1 that at 0° and in low concentration, α -*d*-mannopyranose, β -*d*-mannopyranose and the equilibrium mixture of *d*-mannose are fermented at a minute rate only. After a fermentation period of 24 min., about 76% of the

Table 1. *Anaerobic consumption of d-mannopyranose and d-glucopyranose by baker's yeast at 0°*

(Substrate concentration: 0.005M. Fermentation period: 24 min.)

Substrate	Hexose added (mg.)	Hexose recovered from suspending fluid (mg.)	Hexose disappearing from suspending fluid (as % of that added)
β - <i>d</i> -Mannopyranose	16.2	12.6	22.2
α - <i>d</i> -Mannopyranose	16.4	12.2	25.6
<i>d</i> -Mannose (equil. mixt.)	16.8	12.8	23.8
α - <i>d</i> -Glucopyranose	16.4	0	100

added mannose may be recovered from the suspending fluid; even if it is assumed that at the end of the experiment the concentration of *d*-mannose inside the yeast cell is not more than 0.001M, only 2.5 mg. of *d*-mannose are consumed, compared with 16 mg. of α -*d*-glucopyranose under otherwise similar conditions.

The results summarized in Table 2 demonstrate the effect of various substrate concentrations on the fermentation rate of *d*-mannose (equilibr.) and of

Table 2. *Rates of fermentation of d-mannose and d-glucose by brewer's yeasts at 10° and at 25° with various substrate concentrations*

No.	Yeast		Substrate concentration (M)	Fermentation rate (ml. CO ₂ /5 min.)		Ratio of fermentation rates (mannose glucose)
	Type	Amount (g.)				
				With <i>d</i> -mannose*	With <i>d</i> -glucose*	
(a) Temperature 10°						
1a†	Top (ale)	6	0.026	0.47	1.04	0.45
2a	"	6	0.130	1.17	1.61	0.73
3a	"	6	0.013	0.36	0.90	0.40
3b	"	6	0.026	0.49	1.10	0.45
3c	"	6	0.106	1.03	1.51	0.68
3d	"	6	0.252	1.36	1.59	0.86
4a	Bottom	5	0.026	0.42	0.96	0.44
5a	"	5	0.026	0.40	0.93	0.43
5b	"	5	0.252	1.11	1.28	0.87
6a	"	5	0.026	0.41	0.94	0.44
6b	"	5	0.130	0.95	1.27	0.75
(b) Temperature 25°						
1b†	Top (ale)	1	0.013	0.79	1.00	0.79
1c	"	1	0.026	1.04	1.19	0.87
1d	"	1	0.106	1.31	1.39	0.94
2b	"	1	0.130	1.93	2.06	0.94
7a	"	1	0.026	1.25	1.64	0.76
7b	"	1	0.130	1.77	1.92	0.92
4b	Bottom	1	0.026	0.92	1.15	0.80
8a	"	1	0.026	0.76	1.02	0.75
8b	"	1	0.252	1.26	1.46	0.86
6c	"	1	0.026	0.87	1.05	0.83
6d	"	1	0.130	1.19	1.38	0.86
6e	"	1	0.252	1.26	1.45	0.87

* The sugar solution was allowed to equilibrate before the experiment.

† Experiments denoted by the same number were carried out within a few hours with portions of the same yeast-suspension freshly prepared for the series.

d-glucose (equilibr.) at 10 and at 25°. From the experimental data the ratio

$$\frac{\text{fermentation rate of } d\text{-mannose}}{\text{fermentation rate of } d\text{-glucose}}$$

has been calculated, and its variation with substrate concentration and temperature is shown in the last column of Table 2. At 10° and in low concentration, *d*-mannose is fermented by brewer's yeast at less than half the rate of *d*-glucose; at higher concentration the difference between the fermentation rates of the two epimeric hexoses becomes much smaller. At 25° and low concentration, the fermentation rate of *d*-mannose may be only 20–25 % less than that of *d*-glucose, whereas in higher concentration, *d*-mannose is fermented 10–15 % slower than *d*-glucose. The results obtained with brewer's top and bottom yeasts are very similar.

Table 3. Rate of fermentation of α - and β -*d*-mannopyranose by brewer's top yeast at 10°

(Initial substrate conc. 0.026M.)

Substrate	Rate of fermentation (ml. CO ₂ /5 min.)	
	Exp. 1	Exp. 2
β - <i>d</i> -Mannopyranose	0.28	0.39
α - <i>d</i> -Mannopyranose	0.33	0.45
α - <i>d</i> -Glucopyranose	0.88	1.00

In Table 3 the fermentation rates of α -*d*-mannopyranose, β -*d*-mannopyranose and α -*d*-glucopyranose by brewer's yeast at 10° are compared.

Table 4. Maximum rates of fermentation of *d*-mannose and of *d*-glucose by extract of brewer's bottom yeast at 10° and at 25°

Preparation and extraction of the dried yeast	Temp. of exp.	Substrate conc. (M)	Max. rate of fermentation (ml. CO ₂ /4 min.)		Ratio of fermentation rates (mannose/glucose)
			<i>d</i> -mannose	<i>d</i> -glucose	
Dried at 28° (48 hr.), extracted at 25° (4 hr.)	10°	0.025	1.08	1.08	1.00
	10°	0.025	1.02	1.07	0.95
	25°	0.050	5.00	5.14	0.97
Dried at 30° (48 hr.), extracted at 35° (2 hr.)	25°	0.050	2.95	4.62	0.64

When fermentation is effected by cell-free yeast extract prepared at temperatures not exceeding 28°, the marked difference in the fermentation rates of *d*-mannose and *d*-glucose, observed when these sugars at low concentration are fermented at 10° by living yeast, disappears completely (Table 4). (See also Fig. 1.)

DISCUSSION

From the results obtained at 10° with living yeast, it may be concluded that both α - and β -*d*-mannopyranose are fermentable without preliminary interconversion by mutarotation. Thus, in Exp. 2,

Table 3, 6.1 and 6.9 mg. respectively of *d*-mannose were consumed within the first 15 min. during which period 14.0 and 7.0 mg. respectively of β -*d*-mannopyranose and α -*d*-mannopyranose were interconverted to isomerides (cf. Hudson & Sawyer, 1917). The alternative conclusion that only a single *d*-mannose modification, e.g. the α -pyranose form

Fig. 1. Fermentation rates of *d*-mannose and *d*-glucose by yeast extract plotted against time. 25°. Inorganic phosphate 0.04M. Substrate conc. 0.074M.

or *d*-mannofuranose, which latter contributes a small proportion to the equilibrium mixture, might be suitable for fermentation can be ruled out by considering the substrate concentrations. A comparison of the fermentation rates of α - and β -*d*-mannopyranose seems to indicate a slight preference for the α -form by baker's and brewer's yeasts

(Tables 1 and 3). In these features *d*-mannose parallels *d*-glucose.

A marked difference, however, between mannose and glucose in their behaviour towards yeast is revealed by the finding (Table 2) that at 25° the ratio $\frac{\text{fermentation rate of } d\text{-mannose}}{\text{fermentation rate of } d\text{-glucose}}$ varies only to a small extent with substrate concentration, whereas at 10° this ratio is greatly reduced with decreasing substrate concentration. This unusual dependency on substrate concentration of the temperature coefficient of mannose fermentation by yeast is

clearly demonstrated by Exp. 6, Table 2. From the figures recorded it may be calculated that the ratios of the velocity of fermentation at 25° to that at 10° (V_{25}/V_{10}) are 6.2 and 10.6 for 0.130M and 0.026M-*d*-mannose respectively; the corresponding figures for *d*-glucose are 5.5 and 5.6. The further observation that the striking difference in the fermentation rates of *d*-mannose and *d*-glucose in low concentration at 10° is found only with intact yeast cells and not with yeast extract strongly suggests that the cell membrane is responsible for this phenomenon. It would appear that *d*-mannose on entering the cell, i.e. at the water-lipid interface, meets a higher energy barrier than does *d*-glucose. With high substrate concentrations at low temperatures differences between *d*-mannose and *d*-glucose with respect to the number of molecules possessing the minimum kinetic energy necessary to overcome the corresponding energy barriers at the interface (activated molecules) do not greatly affect the ratio of their fermentation rates, because in each case the number of molecules penetrating the cell membrane will be sufficient to saturate or nearly to saturate the enzyme catalyzing the initial fermentation reaction. With low substrate concentrations, however, differences between *d*-mannose and *d*-glucose in regard to the number of activated molecules will have a marked effect on the ratio, since under these conditions the rate of the initiating reaction is very sensitive to a change in substrate concentration. Increase in temperature from 10 to 25° greatly increases the proportion of activated molecules. This accounts satisfactorily for the experimental result that at 25° the ratio

$$\frac{\text{fermentation rate of } d\text{-mannose}}{\text{fermentation rate of } d\text{-glucose}}$$

varies much less with substrate concentration over the whole range tested than it does at 10°. The finding that at 0° and 0.005M substrate concentration the fermentation rate of mannose is only a fraction of that of glucose (Table 1) is in good agreement with this interpretation.

In this connexion attention may be drawn to an observation made by Leibowitz & Hestrin (1939). In experiments with living brewer's yeast the ratio of the fermentation rate of methyl- α -glucoside to that of *d*-glucose was found to be 0.24 at 35°, 0.033 at 25° and $1/\infty$ at 4°. Maltose, on the other

and maltose had already reached their maxima. With maceration juice of brewer's yeast, however, the maximum fermentation rates of glucose, maltose and methyl- α -glucoside were identical both at 28° and at 4°, even at 2% substrate concentration. Leibowitz & Hestrin supposed that the fermentation paths of methyl- α -glucoside and maltose are different, the former being fermented only after hydrolysis by maltase, to which they ascribed an extremely high temperature coefficient, and the latter being fermented directly. Taken with our results this work would suggest that the extraordinarily high temperature coefficient of the fermentation of methyl- α -glucoside by living yeast is due to the high energy barrier encountered by the glucoside at the water-lipid interface (cf. Kozawa, 1914). The observations of Leibowitz & Hestrin and in the present paper are in harmony with the finding of Jacobs, Glassman & Parpart (1935) that the temperature coefficients of haemolysis of mammalian erythrocytes are high in the presence of non-electrolytes which only slowly penetrate the cell membrane.

Equality of the maximum fermentation rates of *d*-mannose and *d*-glucose by yeast extract at 25° and at 10° was observed only when the yeast is dried at a temperature not exceeding 28° and then extracted at 25°. With higher temperatures the rate of mannose fermentation was invariably lower than that of glucose (Table 4). This is in conformity with earlier findings of Slator and of Jephcott & Robison. Slator (1908) reported that exposure of living yeast to heat diminishes its capacity to ferment mannose to a much greater extent than its capacity to ferment glucose. Jephcott & Robison (1934) (cf. also Neuberg & Ostendorf, 1930) discovered that mannose-6-phosphate, formed when brewer's yeast, dried at 37° for 24 hr., acts upon *d*-mannose at 38°, may account for almost the whole of the hexose monophosphate fraction and more than 80% of the total ester P. With *d*-glucose 80% of the phosphorylated products are represented by fructose diphosphate and only 20% by the monophosphate fraction, the latter consisting mainly of a mixture of fructose-6-phosphate and glucose-6-phosphate together with minute amounts of mannose-6-phosphate. These results suggest that the conversion of the individual aldo-hexose-6-phosphate to the common intermediate fructo-

hand, was fermented at practically the same rate as glucose at the various temperatures. At the substrate concentration used (10%) the fermentation rate of methyl- α -glucoside was still markedly dependent on concentration, while those of glucose

furanose-6-phosphate is catalyzed by two specific hexose phosphate isomerases, isomerase II being more readily destroyed between 28 and 38° than isomerase I. More evidence, however, is necessary to prove the existence of the two isomerases.

Recently, Berger, Slein, Colowick & Cori (1946) and Kunitz & McDonald (1946) have shown that the hexokinase reaction proceeds with mannose at about half the rate with *d*-glucose. This may account for the longer time taken to reach the maximum rate in the fermentation by yeast juice at 25° of mannose as compared with glucose (cf. Fig. 1). The equality of the two maxima is best explained by the previous observation that at low phosphate concentration (0.040M) the reaction

controls the over-all rate of hexose fermentation by dried yeast (Gottschalk, 1946).

It would thus appear that the observed differences between the fermentation of *d*-mannose and *d*-glucose by yeast cells and by yeast preparations over the range of temperature tested (0–38°) may be due to: (1) the slower rate at which mannose penetrates the cell-membrane, and/or (2) the slower rate at which mannose reacts with hexokinase, and/or (3) the instability of mannose-6-phosphate isomerase at temperatures above 28°.

SUMMARY

1. In contrast to that of *d*-glucose, the temperature coefficient of *d*-mannose fermentation by yeast cells is dependent on substrate concentration; it increases considerably with decreasing mannose concentration. With yeast extract as fermenting agent no such difference between mannose and glucose is observed. It would thus appear that the cell membrane is responsible for this phenomenon.

2. The maximum rates of mannose and glucose fermentation at 25° by yeast extract are the same provided that during the preparation of the yeast extract the temperature does not exceed 28°; with higher temperatures mannose is fermented markedly slower than glucose. Taken with previous findings of Jephcott & Robison (1934) this result suggests the existence in yeast of two aldo-hexose phosphate isomerases, the mannose-6-phosphate isomerase being more readily destroyed at 28–38° than the glucose-6-phosphate isomerase.

3. Experiments at 10° afford evidence that both α - and β -*d*-mannopyranose are fermented without preliminary interconversion by mutarotation.

REFERENCES

- Berger, L., Slein, M. W., Colowick, S. R. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.
 Gottschalk, A. (1943*a*). *Aust. J. exp. Biol. med. Sci.* **21**, 133.
 Gottschalk, A. (1943*b*). *Aust. J. exp. Biol. med. Sci.* **21**, 139.
 Gottschalk, A. (1945). *Aust. J. exp. Biol. med. Sci.* **23**, 261.
 Gottschalk, A. (1946). *Biochem. J.* **40**, 621.
 Gottschalk, A. & Rawlinson, W. A. (1942). *Aust. J. exp. Biol. med. Sci.* **20**, 169.
 Hill, A. V. (1930). *Proc. Roy. Soc. B*, **106**, 477.
 Hopkins, R. H. (1931). *Biochem. J.* **25**, 245.
 Hudson, C. S. & Sawyer, H. L. (1917). *J. Amer. chem. Soc.* **39**, 470.
 Jacobs, M. H., Glassman, H. N. & Parpart, A. K. (1935). *J. cell. comp. Physiol.* **7**, 197.
 Jephcott, C. M. & Robison, R. (1934). *Biochem. J.* **28**, 1844.
 Kozawa, S. (1914). *Biochem. Z.* **60**, 231.
 Kunitz, M. & McDonald, M. R. (1946). *J. gen. Physiol.* **29**, 393.
 Kuttner, T. & Cohen, H. R. (1927). *J. biol. Chem.* **75**, 517.
 Leibowitz, J. & Hestrin, S. (1939). *Enzymologia*, **6**, 15.
 Levene, P. A. (1924). *J. biol. Chem.* **59**, 129.
 Neuberg, C. & Gottschalk, A. (1925). *Biochem. Z.* **161**, 244.
 Neuberg, I. S. & Ostendorf, C. (1930). *Biochem. Z.* **221**, 154.
 Slator, A. (1908). *J. chem. Soc.* **93**, 217.
 Sobotka, H. & Reiner, M. (1930). *Biochem. J.* **24**, 394.

The Effect of Diet on the Glutathione Content of the Liver

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It has been shown recently (Glynn, Himsworth & Neuberg, 1945) that white rats kept on a diet containing all the amino-acids occurring in proteins with the exception of methionine and cystine, develop after varying lengths of time massive necrosis of the liver. Although it is not quite certain that the dietary deficiency of sulphur-containing amino-acids was the sole cause of the liver lesions observed, the results suggested that the proneness of the rats to develop hepatic necrosis was speci-

fically associated with a lack of cystine, or its potential precursor methionine, in the amino-acid mixture. It seemed likely that the absence of these amino-acids from the diet would produce a reduction in the relative concentration of the non-protein sulphur in the tissues. Thus Lee & Lewis (1934) found in rats, which had been reared on a diet relatively deficient in cystine, low sulphur values in protein-free extracts of liver, kidney and muscle; these authors also observed that the ratio of