XV. THE KINETICS OF ALCOHOLIC FERMENTATION OF SUGARS BY BREWER'S YEAST.

IV. SPECIFICITY. THE RATES OF FERMENTATION OF α - AND β -GLUCOSE.

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ARMSTRONG [1904] advanced the hypothesis that the specificity exhibited by yeast towards glucose, mannose and fructose was due to the existence of an enol form common to the three hexoses. Hopkins [1931] developed a modification of this hypothesis to show certain features of configuration common to α -glucopyranose, α -mannopyranose and fructofuranose using the configurations of Haworth. In the case of glucose, Willstätter and Sobotka [1922] and Hopkins [1931] had shown that, when a solution of glucose is fermented rapidly by brewer's yeast and the fermentation is interrupted, the residual solution exhibits mutarotation in the positive sense. This was interpreted to mean that the yeast selectively preferred the α -form of glucose. Hopkins [1931] had further shown that fructose, partly fermented by brewer's yeast, also exhibited mutarotation in the positive sense. The inference was drawn that brewer's yeast specifically ferments a form of fructose possessing a less negative specific rotation than that of fructose at equilibrium; this was quite possibly fructofuranose which shows a certain configurational relationship with glucopyranose and mannopyranose.

The simplest explanation of these phenomena is to suppose that brewer's yeast specifically ferments α -glucose, α -mannose (for which the evidence will be furnished in a later communication) and some form of fructose. However, there is the alternative explanation that, whilst both α - and β -forms of the sugars are directly fermentable, the α -form is preferred just as glucose is preferred to fructose in mixtures of the two. Certain experiments of Willstätter and Sobotka [1922] seem to support this, in that α - and β -glucose separately fermented at the same rate, just as glucose and fructose do. However, these fermentations were conducted at concentrations high enough to prevent concentration of sugar from being the limiting factor. Lastly there remains the possibility that intermediate forms of the sugars are specifically attacked.

In recent years evidence has been forthcoming to strengthen this last hypothesis. Lippich [1932] measured the HCN-binding powers ("Blausäurezahl") of sugar solutions and concluded that open-chain forms were present in appreciable amounts. One or, more probably, two such "N-forms" may exist. Thus for glucose he suggests either

the N_A and N_B forms being presumably open-chain forms related stereochemically in some such manner as are α - and β -glucose. Although Lippich was forced to

consider (2) to be more representative of a glucose solution, it is simpler for our purposes to make use of (1), or of a modification of (2) in the form,

the N_A form being that specifically fermented by brewer's yeast.

With a view to deciding between the three explanations outlined above of the specificity of brewer's yeast for glucose it was considered desirable to measure the rates of fermentation of α -, β - and $\alpha\beta$ -glucose, all at concentrations low enough to influence or even to control the rate of fermentation.

EXPERIMENTAL.

The apparatus used for measuring velocities of fermentation was that used by Slator [1906] with modifications and technique as described by Hopkins and Roberts [1935, 1]. In the fermentation vessel the brewer's yeast in suspension was brought to the desired temperature and the sugar, in solid form or in solution at equilibrium, was contained in a small beaker on the float on the surface. This sugar was discharged into the yeast suspension by starting the shaker after exhaustion of the system. α -Glucose was recrystallised from alcohol, dried *in vacuo* over P_2O_5 and finally desiccated in a Fischer drier. β -Glucose was crystallised from pyridine, dried and freed from pyridine at 100° for 2 hours, its identity being confirmed by polarimetric observations.

The rates of fermentation were observed by reading the manometer at intervals of 0.5–2 min. and calculated to mg. CO_2 /min. using the appropriate calibration factor for the total volume and temperature of liquid employed [Hopkins and Roberts, 1935, 1].

The results in Tables I and II show that α -, β - and $\alpha + \beta$ -glucose at 1 % concentration are fermented at 30° at the same rate. However, in the case of $\alpha + \beta$ -glucose in equal proportions, rather less time was required to attain

Table I. *Rates of fermentation of α - and β -glucose.*

1 g. α - or β -glucose; 4 g. yeast; 100 ml.; 30° ; CO_2 as mg./min.

Time (min.) ...	2	5	10	15	20	25	30	35
α -glucose	3.40	4.98	5.29	5.23	5.18	5.12	4.93	4.75
β -glucose	3.40	4.95	5.32	5.23	5.20	5.15	4.93	4.75

Table II. *Rates of fermentation of (1) α -glucose, (2) an equimolar mixture of α - and β -glucose.*

(1) 0.5 g. α -glucose, (2) 0.25 g. α -glucose + 0.25 g. β -glucose. 4 g. yeast; 50 ml.; 30° ; CO_2 as mg./min.

Time (min.) ...	1	2	4	6	8	10	15	20
α -Glucose	0.26	1.04	2.08	2.81	3.30	3.74	4.47	4.94
$\alpha + \beta$ -Glucose	0.52	1.30	2.29	3.02	3.53	3.95	4.68	5.10
Time (min.) ...	25	30	35	40	45	50	55	60
α -Glucose	5.20	4.99	4.58	4.06	3.54	2.91	1.56	0.41
$\alpha + \beta$ -Glucose	5.20	4.89	4.37	3.95	3.54	2.81	1.56	0.52

the maximum rate than with α -glucose alone. Subsequent fermentations were carried out at 35° , since it was found that with the very high yeast rate that was necessarily employed the induction period was greatly reduced at this temperature. The results of these later experiments are shown in Figs. 1 and 2.

DISCUSSION.

Selective fermentation of $\alpha\beta$ -glucose, as observed by the mutarotation of the partly fermented sugar, indicates that the α -form is removed more rapidly than the β -form [Willstätter and Sobotka, 1922; Hopkins, 1931]. The results however do not support the simple hypothesis that brewer's yeast is specific for α -glucose. For example, calculation shows that in the case of β -glucose at 35° and p_H 5.0–6.0 (Fig. 1) the 1% solution would after 6 min. unmolested mutarotation have yielded about 0.11% of α -glucose. At the same stage the α -glucose (Fig. 1) would have produced about 0.22% of β -glucose, leaving 0.78% of α -glucose unchanged. If the yeast were specific for the α -form, the rate of fermentation of the former sugar solution at this stage would be the same as that of $\alpha\beta$ -glucose containing 0.11% of the α -form, *i.e.* 0.30% $\alpha\beta$ -glucose, and the rate for the latter solution the same as that of 2.1% $\alpha\beta$ -glucose. The latter would show the greater rates of fermentation if the yeast were in any way specific for the α -form. However, after 6 min. the maximum rates of fermentation were attained in each case and were the same. These experiments prove this equality more clearly than the corresponding ones of Willstätter and Sobotka [1922] who employed much higher concentrations of the sugars and measured the times of half-fermentation.

In these earlier experiments variations in yeast rate and temperature at 1% sugar concentration were introduced. The only difference revealed between the sugars investigated (α -, β -, $\alpha + \beta$ - and $\alpha\beta$ -glucose) was between $\alpha\beta$ -glucose on the one hand and freshly dissolved α - or β -glucose on the other at 35° (Fig. 1).

Fig. 1. Rates of fermentation of α -, β - and $\alpha\beta$ -glucose respectively, at 1% concentration. 0.5 g. α -, β -, or $\alpha\beta$ -glucose; 8 g. yeast; 50 ml.; 35° . α - $\circ$, β - $\square$, $\alpha\beta$ - $\triangle$.

These conditions were adopted since, from a consideration of the temperature coefficient of mutarotation and the observed induction periods, it was apparent that the maximum rate of fermentation would then be attained with minimum mutarotation. The distinctive appearance of the curves (Fig. 1) for α - and β -glucose would seem to be due to the relative velocities of fermentation and mutarotation, since under other conditions (Tables I and II) the curves were of the usual compound exponential type and resembled those of $\alpha\beta$ -glucose in all experiments. The times of half-fermentation are the same in all three cases, *viz.* 12, 11.9 and 12.1 min. for α -, β -, and equilibrium glucose respectively, a result

obtained by Willstätter and Sobotka [1922]. If we assume, as supported by the results of these experiments, that the fermentable form is some intermediate form of the sugar, *e.g.* Lippich's N-form, the initial rise in the rate of fermentation would be due to the high initial concentration of this form which would have accumulated at equal rates from the α - and β -forms during the induction period. Considering the case of α -glucose as initial sugar, the concentration of such in solution is initially 1% and that of the N-form which would establish equilibrium with it would be presumably some definite fraction of this. During the induction period the N-form is removed almost solely by its transformation into the β -form, and its concentration might be higher than that in equilibrium glucose of 1% concentration. Subsequently, with rapid removal of the N-form, mainly by fermentation, conditions having been designed to give a rate of fermentation faster than the rate of mutarotation, the concentration falls and with it the rate of fermentation. The concentration of N-form may be regarded as never exceeding a definite fraction of the concentration of α -form present, and the latter is falling unimolecularly. However, a certain quantity of β -glucose has been formed, mainly during the induction period. A stage is reached when the concentration of the N-form has fallen low enough to permit the back reaction, $\beta \rightarrow N$ -, to come into play and reinforce the supply of N-glucose hitherto drawn solely from the α -glucose. The flattening in the curve is thereby explained. The subsequent course of the fermentation is the usual one. Exactly similar considerations can account for the β -glucose fermentation curve. The equilibrium glucose fermentation curve is of the normal compound exponential type. After the induction period a quasistationary period follows, and later, when the concentration of unfermented glucose has fallen to a certain point, an exponential fall. The curve differs from those of α - and β -glucose because of the assumed initial excessive production of N-glucose in the latter cases. That the maximum velocity of fermentation attained during this excessive production is not much higher than that attained by equilibrium glucose is not surprising in view of the relative velocities of fermentation of glucose solutions in the neighbourhood of 1% concentration [see Hopkins and Roberts, 1935, 1].

The explanation advanced above of the curves for α - and β -glucose fermentation can be amplified by the following mathematical treatment.

Consider the reactions,

fermentation products

each reaction proceeding unimolecularly.

Let k_1 = velocity constant of reaction (1) and k_2 = velocity constant of reaction (2) including the fermentation and change to β -glucose.

Let a = initial concentration of α -glucose, then $\frac{dx}{dt} = k_1(a - x)$ where x = concentration of α -glucose removed in time t , *i.e.*

$$x = a(1 - e^{-k_1 t}) \quad \dots\dots(1).$$

Also $\frac{dy}{dt} = k_2(x - y)$ where y = concentration of N-glucose removed in time t ,
 $= k_2[a(1 - e^{-k_1 t}) - y],$

$$\frac{dy}{dt} + k_2 y = a k_2 (1 - e^{-k_1 t}).$$

Integration and evaluation of the constant arising from it gives

$$y = a - \frac{a k_2}{k_2 - k_1} e^{-k_1 t} + \frac{a k_1}{k_2 - k_1} e^{-k_2 t} \quad \dots\dots(2).$$

The concentration of N-glucose at time t is $(x - y)$, and from equations (1) and (2)

$$x - y = \frac{ak_1}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t}).$$

By differentiation, this expression is found to have maximum value when

$$t = \frac{\log_e k_1 - \log_e k_2}{k_1 - k_2} = T,$$

and to show a point of inflection when

$$t = 2 \frac{\log_e k_1 - \log_e k_2}{k_1 - k_2} = 2T.$$

On the assumption that α -glucose produces N-glucose unimolecularly, and that the latter in its turn is removed unimolecularly, the concentration of N-glucose will rise to a maximum and later show a definite fall. This is in agreement with the shape of the curves until the rapid fall in rate of fermentation has set in.

Alternatively, if it be assumed that reaction (2) proceeds for a time at a constant rate, limited by a constant rate of fermentation, then $\frac{dy}{dt}$ becomes constant and $= k_2$ (say) whereupon the concentration of the N-form, $x - y = a(1 - e^{-k_1 t}) - k_2 t$, which rises to a maximum value at time $t = \frac{1}{k_1} \log_e \frac{ak_1}{k_2}$, and thereafter declines. Again the concentration of the N-form rises to a maximum and then declines.

That abnormal transformations are taking place during the very early stages of mutarotation of either α - or β -glucose to equilibrium is supported by the physical observations of Riiber and Minsaas [1926].

To summarise, if we assume that α -glucose yields β -glucose, and that β -glucose yields α -glucose, either wholly or partly *via* an intermediate form, and that each individual reaction is unimolecular, then the curves for the fermentations of α - and β -glucose in Fig. 1 can be explained. No other explanation has been found which seems feasible. This is evidence that neither α - nor β -glucose is specifically fermented, but that some other form into which they change is so.

As regards the nature of the intermediate form specifically fermented there is no need to postulate what this may be. The simplest assumption however is that it is the aldehyde form. According to Lippich's [1932] conclusions, there should be a sufficiency of the aldehyde form present in $\alpha\beta$ -glucose. In view of the theoretical deductions of Slator and Sand [1910] that 0.43 mg./l. represents the difference in concentration of sugar (which we must now postulate as the specifically fermentable component) outside and inside the cell when stirring is maintained, the 0.25 % of glucose present as aldehyde (Lippich) should be adequate in concentration to promote fermentation even when the total sugar concentration is as low as 0.018 %.¹

The observations on the mutarotation of partly fermented $\alpha\beta$ -glucose also may find explanation in the specific fermentation of some other form, *e.g.* aldehyde or one of Lippich's N-forms. If we assume for convenience that at equilibrium glucose contains 35 % of the α - and 64 % of the β -form (the usually accepted proportions), together with a small proportion of the N-form, we may regard the 35 parts of α -form as producing a unit quantity of the β -form by way of the N-form in the same time as the 64 parts of β -form produce a unit quantity of the α -form, the equilibrium being thus maintained. During fermentation this N-form of glucose is removed, whereupon it is restored by equal contributions from the α - and β -forms. But for equilibrium during fermentation the α - and β -forms should have contributed to the N-form in the proportions 35 : 64. In other

¹ It is significant that Hopkins and Roberts [1935, 1] found that with glucose at concentrations of this order the fermentation followed a unimolecular course.

Fig. 2 (a, b, c). Rates of fermentation of α - and β -glucose at different concentrations. 8 g. yeast; 50 ml.; 35°.

	a	b	c
α -Glucose (o)	0.252 %	0.508 %	0.753 %
β -Glucose (•)	0.255 %	0.501 %	0.749 %

words, the β -form is not contributing its share, so that the proportion of α - and β -forms becomes changed to one with an abnormally high proportion of β -glucose. On arresting the fermentation, mutarotation to equilibrium follows, as observed. Some evidence in support of this explanation is furnished in Fig. 2, which is discussed below. Alternatively, the changes of rotation in partly fermented glucose solutions can be accounted for by postulating merely that the specific rotation of the N-form is higher than that of $\alpha\beta$ -glucose. The removal of this form would have the effect observed. If however the N-form is present in only a relatively low proportion, then its specific rotation must be high. Both of these explanations may apply to the case, the resultant of the two effects being the mutarotation observed.

The above explanation of the phenomenon of selective fermentation of $\alpha\beta$ -glucose assumes, as stated, that 35 parts of the α -form would in a given time produce the same amount of the N-form as would 64 parts of the β -form. If this is so, then, if sufficiently low concentrations are employed, α -glucose should ferment separately faster than β -glucose. A concentration of 1% is not low enough, but Fig. 2 (*a, b, c*) clearly shows this effect, progressively increasing as concentration is lowered from 0.75 to 0.25%. Application of the Michaelis and Menten theory as described by Hopkins and Roberts [1935, 1], and plotting the reciprocals of maximum velocity as ordinates against reciprocals of initial concentration, yielded the graphs in Fig. 3. From these, following the procedure described by Hopkins and Roberts [1935, 2], the following values of K_m , the Michaelis and Menten constant, were calculated:

α -glucose, 0.178 corresponding to 0.0099 *M*.

β -glucose, 0.212 corresponding to 0.0118 *M*.

That the ratio of these, 0.21 : 0.18, is not as great as that of 64 : 35 is not surprising since, firstly, the observed maximum velocities were attained only after a certain amount of mutarotation had taken place, secondly, the ratio 64 : 35 for

Fig. 3. α -glucose $\circ$, β -glucose $\bullet$.

the proportions of β - and α -glucose respectively in solutions at equilibrium may be far from correct, and thirdly, experimental errors are appreciable. Nevertheless, the results of Figs. 2 and 3 support the former of the two explanations furnished to account for the phenomenon of the mutarotation of partly fermented $\alpha\beta$ -glucose.

SUMMARY.

1. The maximum rates of fermentation attained by α -, β - and $\alpha\beta$ -glucose are the same unless the initial concentration of sugar is well below 1 %.
2. With initial concentrations of 0.5 and 0.25 % the fermentation of α -glucose attains a maximum rate greater than that attained in the case of β -glucose.
3. The values of the Michaelis and Menten constant, K_m , for fermentation of α - and β -glucose are 0.0099 and 0.0118 M respectively.
4. These observations, and particularly the progress of the fermentations at 1 % concentration, lead to the hypothesis that neither α - nor β -glucose, but an intermediate, possibly open-chain, form of glucose is specifically fermented. The α -form is converted into this fermentable form more rapidly than the β -form.
5. The mutarotation of partly fermented $\alpha\beta$ -glucose may be explained on this hypothesis.

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