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On the Rôle of Adenylpyrophosphatase in Alcoholic Fermentation and on the Occurrence of Trehalose during Fermentation with Maceration Juice *

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The differences between the course of the fermentation of the sugar in the living yeast cell, on the one hand, and in zymase preparations, on the other, have for many years been the object of exhaustive studies at this Institute. By using dried preparations of bottom yeast, which have been produced so cautiously that the entire glycolytic enzyme system of the living yeast cell has remained intact, while the permeability hindrances in the cell wall have been removed, it has been possible to place the experiences from the maceration juice fermentation in relation to the fermentation conditions in the living cell.

On the basis of the investigations a comprehensive series of treatises [1—10] have been published. The two most important results of those therein reported will be dealt with in this paper.

In what way does the zymase system of the living yeast cell differ from that of the maceration juice?

The living yeast cell contains a fermentation principle, presumably a dephosphorylating enzyme, which is wanting in yeast maceration juice, and

* This paper was received by me July 21st, 1948, for publication in the *Archives of Biochemistry*. By agreement with professor Karl Myrbäck it was intended to precede a paper by Elander and Myrbäck: »Isolation of Crystalline Trehalose after Fermentation of Glucose by Maceration Juice», with which it is connected. In consequence of certain proposals from the American Editor, with which the authors, from different reasons, could not comply, the paper by Nilsson and Alm is presented here. The paper by Elander and Myrbäck will appear in the *Archives of Biochemistry*.

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which is essential for the rapid fermentation of the entire amount of sugar added. For a detailed discussion of this phenomenon, the reader is referred in particular to a recapitulatory lecture given in 1942 [10] *.

With the result briefly related above are connected the investigations recently published by Meyerhof [13, 14], concerning the rôle of adenylpyrophosphatase in fermentation. It is, however, incorrect, when Meyerhof considers that he is able to explain all our observations in the following way [13, p. 118]. »All observations of Nilsson are easily explained by the greater or smaller destruction of the yeast apyrase in his different preparations.» This should soon be quite clear to anyone taking the trouble to study our communications. We will first discuss Meyerhof's view of the different periods in fermentation. In the publication [13, pp. 117 and 118] already cited, he writes: »The most extensive work devoted to this problem is that of Nilsson, who, between 1936 and 1942, investigated yeast preparations which either showed the usual break when half of the sugar was fermented or did not show it (intakte Trockenhefe). In the latter case addition of cytolytic agents, dyestuffs, *etc.*, induced the appearance of this break.»

The break in the fermentation curve that is induced by the interventions in question, does not appear after half of the sugar has been fermented to alcohol and carbon dioxide, but when an amount of CO_2 has been produced which, according to Harden's equation, corresponds to the amount of free phosphate at the beginning of the fermentation [6]. What takes place between this break and the break occurring later when half of the sugar is fermented, constitutes a period in the fermentation process with which Meyerhof is unfamiliar. We will return to this later in this paper (pp. 216 and 229).

The problems that our publications have introduced into the literature dealing with alcoholic fermentation can in the main only partially be further elucidated by experiments made in fermenting mixtures of the kind used by Meyerhof and with the methods employed by him. The differences in the arrangement of the experiments are much too great. Thus, for instance in Meyerhof's experiments, the substrate/enzyme ratio is incomparably smaller than in our experiments. We have already shown [2, p. 391] what the consequences of this can be. Further, Meyerhof studies the rate of fermentation during various sections of time, whereas we follow the entire fermentation process from beginning to end.

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* The same theme was also treated in a lecture given on April 27th, 1946, at Helsinki University. Here special emphasis was laid upon certain facts affording support for the view that the new enzyme is of general significance for the normal metabolism of all living cells. Evidence in favour of this is supplied, *inter alia*, by the investigations made by Virtanen *et al.* [11, 12] into the formation of lactic acid in dried preparations of lactic acid bacteria.

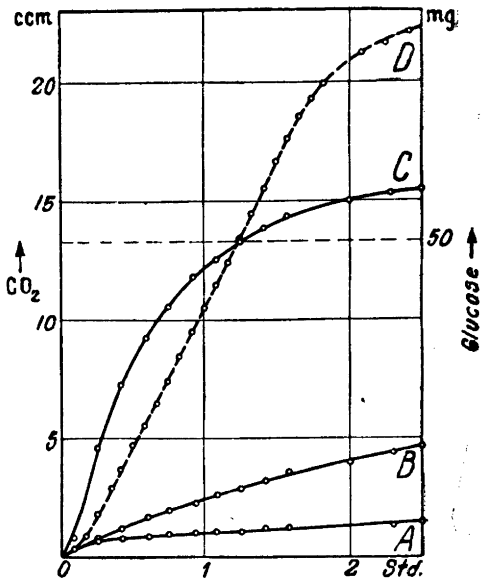


Fig. 1. Vergärung von Hexosediphosphorsäure durch intakte Trockenhefe. Gärtemperatur 30° C.

In the matter of all details we refer the reader to our publications already cited, while we in the following illustrate in a few fundamental respects the differences between Meyerhof's opinion and our own.

It can thus in the first place be pointed out that Meyerhof's experiments provide no explanation whatever of the profound influence exerted upon the fermentation by the agents studied by us, *e.g.* alcohol, toluene, bile acids, *etc.* [6, 8, 10] *.

According to Meyerhof, the slow fermentation period is to be explained exclusively by the fact that the fermentation system lacks a sufficient amount of adenylpyrophosphatase for the fermentation of the hexosediphosphate [14, p. 143]. »If potato enzyme is added to a yeast extract fermenting free sugar at a time where the speed of the phosphate period has fallen down to the low level of the second period, the rate rises again to the level of the phosphate period. *By mere addition of enough purified ATP-ase the fermentation type of yeast extract is changed into the fermentation type of living yeast.*«

One wonders then why, under the same conditions, no fermentation of the accumulated hexosediphosphate can be induced by the addition of intact dried yeast, which completely ferments glucose without the occurrence of

* Meyerhof himself, as we know, even used alcohol in preparing adenylpyrophosphatase from yeast.

any other, slower fermentation period. We reproduce in this connection the following experiment published [10, pp. 29 and 30] by us earlier.

»Dieser Versuch wurde so ausgeführt, dass zunächst in einem Macerationssaft 200 mg Glucose + die äquivalente Menge Phosphat bis zum Knick der Gärkurve vergoren wurden. Die CO_2-Entwicklung hat dann praktisch aufgehört (Kurve A), und die Hälfte des Zuckers (100 mg) liegt als Hexosediphosphorsäure vor. Jetzt wird in einem Gäransatz intakte Trockenhefe zugegeben (Kurve B). Ein zweiter Gäransatz bekommt sowohl intakte Trockenhefe als auch 100 mg Glucose (Kurve C). Ein Vergleich der Kurven B und C zeigt, wie ersichtlich, dass auch in der permeablen, intakten Trockenhefe die unveresterte Glucose mit einer ungleich grösseren Geschwindigkeit vergoren wird als die Hexosediphosphorsäure. Kurve D zeigt die Vergärung von 100 mg Glucose mit intakter Trockenhefe in einem Gäransatz ohne vorangehende Macerationssaftgärung. Die maximale Gärgeschwindigkeit ist hier etwas niedriger als in dem Gäransatz C, was sich durch den Zuschuss an Zymase erklärt, den letzterer Ansatz in Form von Macerationssaft bekommen hat.«

The investigations carried out by Meyerhof with adenylpyrophosphatase obviously offer no explanation of the phenomena here demonstrated.

To the remarkable fact that, as shown by Curve C, glucose added to this system produces only half of the calculated amount of CO_2 with intact dried yeast, we will return later (p. 229).

On the occurrence of a sugar deficit during fermentation in maceration juice and on the chemical character of this deficit

If sugar is fermented with maceration juice * in a fermenting mixture containing a smaller number of moles of phosphate than moles of hexose, two breaks occur in the fermentation curve. The first break occurs when an amount of CO_2 has been evolved that, according to Harden's equation, corresponds to the amount of free phosphate in the fermenting mixture at the onset of fermentation. The second break occurs — irrespective of the amount of phosphate — when half of the sugar has been fermented to alcohol and carbon dioxide [2, pp. 378–382]. In order to illustrate this fact we reproduce the following, previously published [2, p. 381] experiment.

»Besonders schön kommt das verschiedenartige Verhalten der intakten Trockenhefe und des Mazerationssaftes in den bei 40° ausgeführten Gärversuchen zum Vorschein.

* Or with any zymase system whatever that lacks the new fermentation principle mentioned in the foregoing.

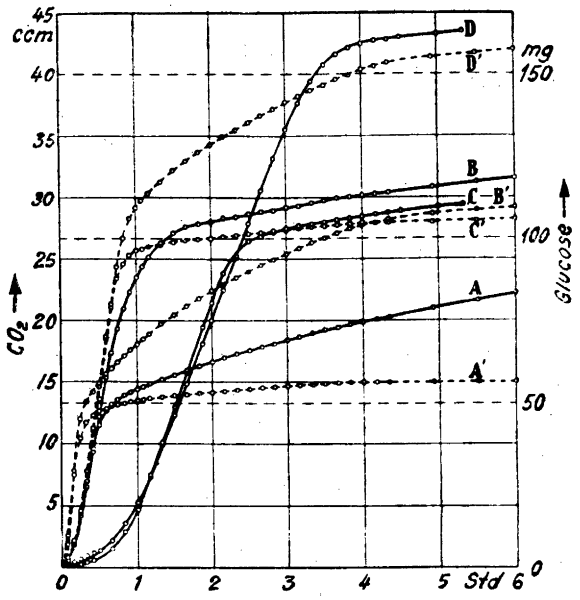


Fig. 2.

Der zu diesem Versuch benutzte Mazerationssaft enthält pro 1 ccm 14.0 mg P_2O_5 (ausschliesslich als Orthophosphat). Der Phosphatgehalt des Mazerationssaftes wird bei der Berechnung der in den Reaktionsmischungen vorhandenen Phosphatmenge berücksichtigt.

Zu jeder Gärungsprobe (Gesamtvolumen 2 ccm): 0.1 ccm Zymophosphatlösung + 1 Tropfen 2.5 %-ige Acetaldehydlösung (in den Proben C und D jedoch kein Zymophosphatzusatz).

In den Proben A, B, C und D 200 mg Trockenhefe H; in A', B', C' und D' 1 ccm Mazerationssaft.

In Probe	A : 100	mg	Glucose	+	Phosphat	äquivalent	mit	100	mg	Glucose
» »	B : 200	»	»	+	»	»	»	100	»	»
» »	C : 200	»	»	+	»	»	»	200	»	»
» »	D : 300	»	»	+	»	»	»	200	»	»
» »	A': 100	»	»	Phosphatgehalt		38.6	mg	P_2O_5		
» »	B': 200	»	»	»		38.6	»	»		
» »	C': 200	»	»	»		79.6	»	»		
» »	D': 300	»	»	»		79.6	»	»		

Reaktionstemperatur 40°.

Bei der intakten Trockenhefe tritt, wie wir schon früher gefunden haben, innerhalb weiter Grenzen unabhängig von der Phosphatkonzentration auf der Gärkurve ein Knick auf, wenn 50 % des Zuckers zu Kohlensäure vergoren sind (Kurve A, B, C und D).

Im Mazerationssaft (Kurve A', B', C', D') ist die Lage des ersten Knickes der Gärkurve durch die *Hardens*che Gleichung festgelegt (aus dem Phosphatgehalt der Reaktionsmischungen berechnet sich nach *Hardens* Gleichung die Lage des ersten Knickpunktes in den Kurven A', B', C' und D' zu 13,1 bzw. 13,1, 26,7 * und 27,0 ccm CO_2). Nach dem Eintreten dieses ersten Knickes schreitet bei Überschuss von Zucker in der Reaktionsmischung die Gärung mit herabgesetzter Geschwindigkeit fort, um noch weiter abzunehmen, etwa wenn der Punkt erreicht wird, wo der Zucker zu 50 % zu Kohlensäure vergoren ist. Ein scharf markierter Knick kann diesmal in Anbetracht der verhältnismässig niedrigen Gärgeschwindigkeit während der zweiten Gärungsphase nicht erwartet werden (Kurve B' und D').»

The phosphate in the fermenting mixture is completely esterified already at the first break and remains esterified during the second, slower fermentation period up to the second break. Nevertheless a transformation takes place during this period in the phosphoric ester (or possibly in the mixture of esters) produced up to the first break. This ester, which is rather difficultly hydrolysable, is gradually transformed into hexosediphosphate, a process that appears to be terminated when the second break is reached. Parallel with this, the content of free sugar in the fermenting mixture decreases and is practically 0 at the second break [7, pp. 66 and 67].

As we have often pointed out in earlier publications [*cf.* 7, pp. 68—70], this peculiar circumstance that we have observed in maceration juice fermentation means that the original amount of sugar in the fermenting mixture cannot, at the second break, be accounted for in the form of known products formed. After deduction of half the amount of sugar, which recurs as alcohol and carbon dioxide, and the amount of hexosediphosphate produced, there remains a sugar deficit. In a suitably chosen composition of the fermenting mixture, this deficit can constitute a very considerable part of the amount of sugar originally added.

From our investigations into the chemical character of this deficit, we have hitherto in our publications only briefly mentioned some results. Here we will confine ourselves to the following quotation [7, p. 70]: »Bei dem zweiten Knick der Gärkurve sammelt sich nämlich in der Gärmischung eine phosphor-

* Die Phosphatmenge ist in dieser Reaktionsmischung dem Zucker gegenüber etwas im Überschuss. Die Lage des Knickpunktes wird somit aus der Zuckermenge berechnet.

freie, nicht reduzierende Substanz an, die bei Erhitzung in n H_2SO_4 ein stark reduzierendes Produkt gibt. Nach Versuchen zu beurteilen, die in letzter Zeit zusammen mit M. Elander ausgeführt wurden, findet unter diesen Versuchsbedingungen ausserdem eine Kondensation vom Typus der Acyloinkondensation statt.»

It is not clear to what extent the sugar deficit observed by us can be placed in relation to the formation of a glycogen-like polysaccharide during the fermentation that has earlier been observed by Harden and Young [15] and further studied by Naganishi [16]. The second break occurring in the fermentation curve after half of the sugar has been fermented to alcohol and carbon dioxide, was unfamiliar to these authors, and Harden assumes that the phosphate is esterified in the form of hexosediphosphate already at the first break. (At that time hexosemonophosphate had not been discovered.) Naganishi finds that the polysaccharide synthesis is promoted by the addition of phosphate to the fermenting mixture. The formation of the substance studied by us is suppressed, on the other hand, by the addition of phosphate (See, for instance, Table 2, p. 222).

In order completely to elucidate the question of the chemical nature and mode of formation of the »sugar deficit», we have made comprehensive investigations, over which a very considerable experimental material is available. The problem has been attacked along two different lines. As already mentioned, it has been examined whether the deficit can be explained by means of an acyloin condensation taking place between the first and the second break in the fermentation curve. Although it was possible to establish such a continuous condensation, this was nevertheless not of such magnitude as to enable the deficit to be accounted for in this way, it seemed. The subsequent investigations were therefore entirely concentrated upon the non-phosphorylated, non-reducing substance which, as we had already found, arises during this fermentation period. As has been mentioned, on hydrolysis with N H_2SO_4 this gives rise to a strongly reducing product. It was found that the hydrolysis product is fermented in the same way as glucose. We further established that the non-hydrolysed substance has an optical rotation corresponding to the disaccharide trehalose. The quantitative conditions prevailing at its formation did not appear to us, however, to be sufficiently investigated for it to be considered fully demonstrated that the sugar deficit established by us consists of trehalose. Accordingly our work was continued with the purpose of clarifying this point completely and of ascertaining the mechanism of the formation of the trehalose. Owing to external circumstances beyond our control these fermentation investigations had to be discontinued in 1941 and the question of their continuance left for the time being. While

waiting for the time when the work could be resumed, we refrained from publishing the investigations that had already been made. From a private communication made to us by Professor K. Myrbäck we have, however, learnt that the question of the chemical nature of the sugar deficit has recently been the object of investigation in his laboratory, crystallized trehalose having been isolated from fermenting mixtures of the kind described by us in the foregoing. This causes us now to single out from our research material and to publish certain of our earlier observations with regard to this matter.

Methods. The maceration juice used in the experiments was prepared according to Nilsson and Alm [17]. For each fermentation sample was used 1 ml maceration juice with addition of 0.05 ml of a 2.5 % solution of acetaldehyde and 0.1 ml of a c. 6 % solution of sodium hexosediphosphate to suspend the induction period. Phosphate was added as a mixture of KH_2PO_4 and $Na_2HPO_4 \cdot 2\,H_2O$, pH≃6.3. The amounts of glucose and phosphate added are indicated in each particular case. The total volume of the fermentation samples was 2 ml, unless otherwise stated.

For the purpose of studying the reaction products formed during the process of fermentation, a series (exactly uniform) of fermenting mixtures in 2 ml amounts (unless otherwise stated) was mechanically shaken in a water thermostat and connected to separate gas burettes for determination of the CO_2 evolved. At different points on the fermentation curve 2 fermentation samples (with some few exceptions) were taken out each time and immediately diluted with 10 ml *aq. dest.* and placed in a boiling water bath for 2 minutes. This causes the protein to coagulate, and after filtering a perfectly clear solution is obtained.

For precipitation of inorganic phosphate and phosphoric esters, an aliquot of the clear filtrates was mixed with $BaCl_2$ in slight excess, made alkaline to phenolphthalein, and absolute ethanol added to 50 per cent of the volume of the solution. After filtration clear solutions were obtained.

Table 1. Reducing substance after Ba precipitation.

Curve	Minutes	CO_2 ml	Reducing substance, mg		Difference	Sugar deficit calc. mg
			Before hydrolysis	After hydrolysis		
	0	0	2.22	5.05	2.83	
	0	0	2.13	4.52	2.39	
A	350	1.30	3.33	6.04	2.71	
	350	1.00	3.13	5.97	2.84	
	0	0	101.7	100.1	−0.6	
	0	0	99.8	99.1	−0.7	
B	350	15.25	5.64	24.40	18.76	27.7
	350	15.15	5.54	24.40	18.86	28.1

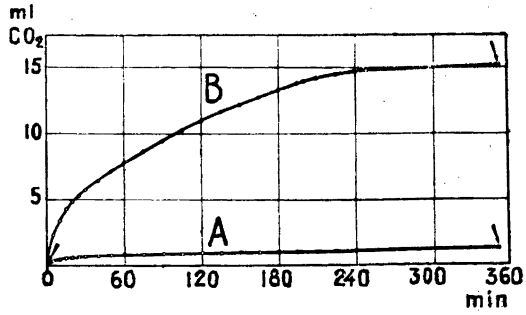


Fig. 3. Fermentation in maceration juice without addition of phosphate. Temp. 40° C.The maceration juice contains an amount of inorganic P equimolar to 30.5 mg of glucose. Curve A: Without additions. Curve B: 100 mg glucose. Analyses in Table 1. (Methods p. 220).

The orthophosphate was determined according to Embden [18], the glucose according to Schaffer-Somogyi [19]. All the reduction values were calculated as glucose and were always computed for the entire reaction mixture (2 ml, unless otherwise stated). The glucose determinations were made partly direct in the filtrate from the Ba precipitation and partly after hydrolysis of the filtrate in *N* H_2SO_4 in a boiling water bath for 3 hours.

From Fig. 3 and the accompanying Table 1, one is inclined to draw the following conclusion. At the second break in the fermentation curve a part of the sugar has been changed into a product (x), which is not itself reducing, but which yields a reducing hydrolysis product (y).

The substance in question (x) does not consist of phosphoric esters, as these were removed with Ba and alcohol before the analyses were made. In the majority of our experiments we have besides satisfied ourselves that the Ba precipitated preparations containing the substance in question (x) were free from inorganic phosphate and after 3 hours' hydrolysis in *N* H_2SO_4 (y)

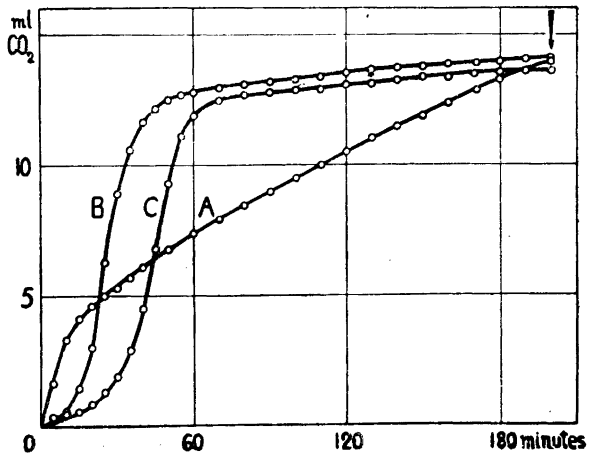


Fig. 4. Fermentation of 100 mg glucose in maceration juice with different amounts of phosphate. Temp. 40° C. The maceration juice contains an amount of inorganic P equimolar to 30.5 mg of glucose. The total amount of inorganic P is in Curves A, B, and C equimolar to 30.5, 80.5, and 130.5 mg of glucose respectively. Analyses in Table 2. (Methods p. 220).

did not give determinable amounts of phosphate. After combustion with $H_2SO_4 - H_2O_2$ it was established that the preparations contained an insignificant amount of total P, not exceeding 1 P in 6 $C_6H_{12}O_6$.

The relation between the hydrolysable substance found (x) and the sugar deficit discussed in the foregoing pages, is illustrated by experiments with varying amounts of phosphate in the fermenting mixture (Fig. 4 and Table 2).

Table 2. Reducing substance after Ba precipitation. Fermentation period 200 minutes.

Curve	CO_2 ml	Reducing substance, mg		Difference	Sugar deficit calc., mg
		Before hydrolysis	After hydrolysis		
A	13.95	3.0	20.4	17.4	32.6
	14.00	3.0	20.4	17.4	32.4
B	14.15	1.3	12.2	10.9	6.9
	14.15	1.2	11.9	10.7	6.9
C	13.55	1.2	5.4	4.2	0
	13.65	1.2	4.7	3.5	0

Even though it was not possible to establish a quantitative agreement, it is nevertheless clearly conceivable that the sugar deficit consists just of the hydrolysable substance found (x).

In order to characterize the substance formed (x), the fermentability of its hydrolysis product (y) was first studied. A larger fermenting mixture, containing per 2 ml 100 mg glucose without addition of phosphate was fermented to the end (Fig. 5, Curve A). After precipitation with Ba and alcohol, and evaporation of the alcohol from the filtrate this was hydrolysed for 3 hours in *N* H_2SO_4 and neutralized. The solution then obtained contained 2.12 mg reducing substance (y) per ml. It is termed preparation 1. The corresponding value before hydrolysis was 0.56 mg per ml. The fermentability of the reducing substance was tested with living brewer's yeast (Fig. 6).

20 ml of preparation 1 contain 42.4 mg reducing substance calculated as glucose.

It appears from Fig. 6 that the hydrolysis product (y) is fermented in exactly the same manner as glucose by living brewer's yeast.

By means of one preparation (Fig. 5, Curve B), made in the same way as in previous experiments, was obtained (after more intensive evaporation) a preparation 2 a and after hydrolysis of a part of this a hydrolysed preparation 2 b. To preparation 2 a was added Na_2SO_4 to the same sulphate con-

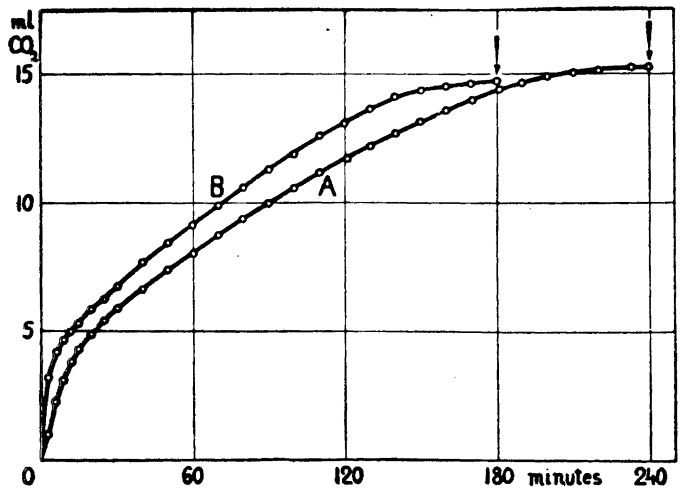
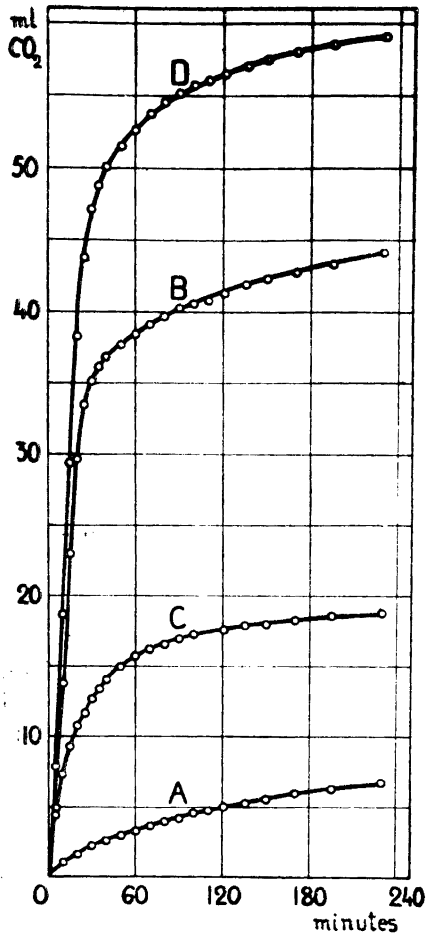


Fig. 5. Control curves of preparations 1, 2 a, and 2 b, p. 222. Temp. 40° C. (Methods p. 220).

centration as in preparation 2 b. The preparations contained 4.05 and 18.38 mg of reducing substance (y) per ml respectively. The fermentability was tested with living brewer's yeast (Fig. 7) as well as with maceration juice (Fig. 8).

The results shown in Fig. 7 confirm those obtained from the experiment just mentioned (Fig. 6) that the reducing substance (y) is fermented in the same way as glucose by living yeast. That this is also the case in fermentation with maceration juice is apparent from Fig. 8. In the latter case, as always in fermentation in maceration juice with a sufficient amount of phosphate, a break occurs when 50 % of the sugar has been converted into alcohol and CO_2.

Judging from the results obtained, the hydrolysable substance found (x) should consist of some kind of condensation product of glucose. In order to gain an idea of the molecular size of this condensation product, its dialysability through a collodion membrane was studied. The preparation used in this case was produced in the way already described. However, the fermenting mixture contained per 2 ml 150 mg of glucose as compared with 100 mg in the earlier experiments. 20 ml of the preparation (solution A) were dialysed in a collodion sack for 19 hours against 40 ml *aq. dest.* The solution outside the collodion sack is termed solution B. The dialysis was continued against running distilled water (13 l) during 24 hours. The remainder in the collodion sack after this time is termed solution C. The reducing substance was determined



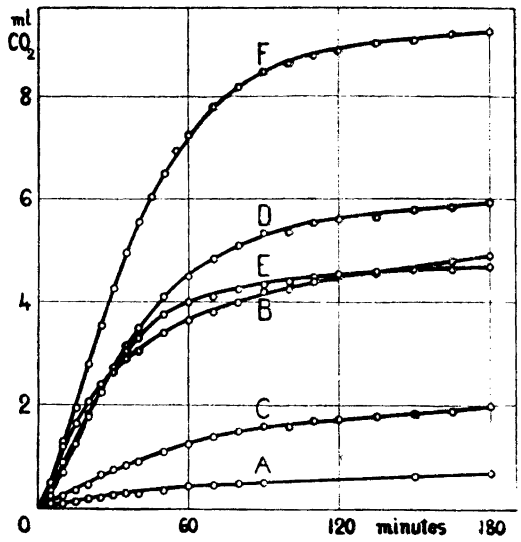


Fig. 7. Fermentation of preparations 2 a and 2 b with living brewer's yeast. Temp. 30° C. To each sample (total volume 2.6 ml) was added 1 ml of yeast suspension (40 g pressed yeast + 100 ml aq. dest.). Curve A: Without additions. Curve B: 20 mg glucose. Curve C: 1 ml of preparation 2 a. Curve D: 1 ml of preparation 2 a + 20 mg of glucose. Curve E: 1 ml of preparation 2 b. Curve F: 1 ml of preparation 2 b + 20 mg of glucose. (Methods p. 220).

Fig. 6. Fermentation of preparation 1 with living brewer's yeast. Temp. 30° C. To each sample were added 3 g of pressed yeast. Curve A: 20 ml aq. dest. Curve B: 20 ml aq. dest. + 200 mg glucose. Curve C: 20 ml preparation 1. Curve D: 20 ml preparation 1 + 200 mg glucose. (Methods p. 220).

in solutions A, B, and C partly before and partly after 3 hours' hydrolysis in N H_2SO_4. The results obtained appear in Table 3, where the total amount of reducing substance (calculated as glucose) in the entire solution is given.

It is evident from the experiment results given in Table 3 that the hydrolysable substance (x) must be low molecular.

In order further to characterize the hydrolysable substance (x), the optical rotation before and after hydrolysis was determined in a solution from the

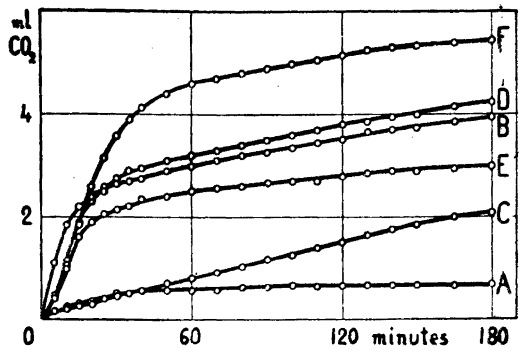


Fig. 8. Fermentation of preparations 2 a and 2 b with maceration juice. Temp. 30° C. To each sample (total volume 2.6 ml) was added 1 ml of maceration juice. Other additions as in Fig. 7. (Methods p. 220).

Table 3. Dialysis experiment.

Solution	Reducing substance, mg	
	Before hydrolysis	After hydrolysis
A	56.8	138.8
B	37.0	112.7
C	0	0

same preparation as was used in the previous experiment. Before hydrolysis the solution already contained a considerable amount of glucose. Through hydrolysis the solution was diluted in the ratio 1 : 2.44. The rotation determinations will be found in Table 4, together with the values for the hydrolysed solution multiplied by the dilution factor.

Table 4. Optical rotation of the hydrolysable substance (x) and its hydrolysis product (y).

Solution	Reducing substance, mg/ml	Optical rotation/dm
Before hydrolysis	2.74	+ 0.64°
After hydrolysis	2.23	+ 0.11°
After hydrolysis corrected with regard to the change in volume	5.44	+ 0.27°

From Table 4 $[\alpha]_D$ can be computed for the hydrolysable substance (x). Of the remaining glucose in the solution there arises an optical rotation of

$52.3° \times 2.74 \cdot 10^{-3} = 0.14°$. The residue, $0.64° - 0.14° = 0.50°$, comes from the substance that after hydrolysis gives in addition $5.44 - 2.74 = 2.70$ mg of reducing substance. The specific rotation for the hydrolysable substance (x) is, assuming it to be a disaccharide, thus: $[\alpha]_D = \frac{0.50°}{2.70} \cdot \frac{360}{342} \cdot 10^3 = 195°$.

This is in good agreement with $[\alpha]_D$ for trehalose, which, according to Schukow [20], has a specific rotation of $[\alpha]_D = + 197.1°$. The specific rotation of the substance obtained after hydrolysis, $[\alpha]_D = \frac{0.11°}{2.23} \cdot 10^3 = 49°$, agrees well with $[\alpha]_D$ for glucose.

In consequence of the results obtained, it now thus seems possible to explain the above-discussed sugar deficit on fermentation in maceration juice by means of the occurrence of a low molecular, non-reducing substance, which has the optical rotation of trehalose and which on hydrolysis yields a product with the optical rotation of glucose, which with living yeast as well as maceration juice is fermented in the same way as glucose.

That the hydrolysable substance (x) consists of trehalose cannot be seriously doubted. The completion of the investigation by the isolation of the trehalose was postponed for the time being, as we felt it to be most important first to obtain a sound basis for judging whether the sugar deficit observed can be entirely traced back to a formation of trehalose. That the trehalose found can exist preformed in the maceration juice in a free state seems to be out of the question in view of the observations made here (pp. 220 and 222). On the other hand, there might be a possibility of the maceration juice containing a trehalose phosphate, which during fermentation transfers its phosphate to the glucose added. What is of importance in this connection is that the maceration juice already on direct hydrolysis without preceding Ba precipitation gives a certain reduction value. This value is of the same magnitude as that obtained after Ba precipitation and hydrolysis at the second break of the fermentation curve when 100 mg of glucose are fermented.

The reducing substance obtained on direct hydrolysis of the maceration juice is, at least partially, fermentable. Our research material is in this respect, unfortunately, insufficient to enable us to form a definite opinion of the significance of these observations. It is, however, clear that a certain cautiousness is not out of place when it is a matter of setting the sugar deficit in relation to the trehalose formed during fermentation.

The sugar deficit in the fermentation is dependent upon the sugar/phosphate ratio at the onset of fermentation and increases if this ratio is increased. If the sugar deficit consists of trehalose, the same must be true for

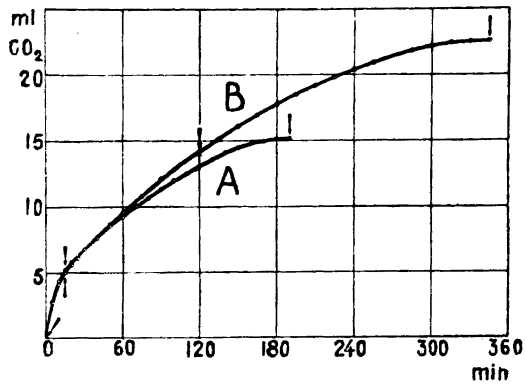


Fig. 9. Fermentation in maceration juice of varying amounts of glucose without addition of phosphate. Temp. 40° C. Curve A: 100 mg glucose. Curve B: 150 mg glucose. Analyses in Tables 5 and 6. (Methods p. 220).

the formation of trehalose during fermentation. How matters stand is illustrated by the following experiment (Fig. 9).

Table 5. Reducing substance after Ba precipitation in the fermentation of 100 mg glucose.

Minutes	CO_2 ml	Reducing substance, mg		Difference	Sugar deficit calc., mg
		Before hydrolysis	After hydrolysis		
0	0	109.5	104.3	— 5.2	
0	0	109.9	104.5	— 5.4	
15	5.55	60.7	64.6	3.9	
15	5.35	60.2	61.1	0.9	
190	15.15	5.3	28.4	23.1	28.1
190	15.10	5.3	28.5	23.2	28.3

Table 6. Reducing substance after Ba precipitation in the fermentation of 150 mg glucose.

Minutes	CO_2 ml	Reducing substance, mg		Difference	Sugar deficit calc., mg
		Before hydrolysis	After hydrolysis		
0	0	154.4	—	—	
0	0	155.1	151.3	— 3.8	
15	5.50	—	—	—	
15	5.10	108.0	99.1	— 8.9	
120	14.25	53.7	69.4	15.7	
120	14.20	55.0	70.7	15.7	
345	22.45	6.2	44.2	38.0	50.8
345	22.55	6.2	43.9	37.7	50.4

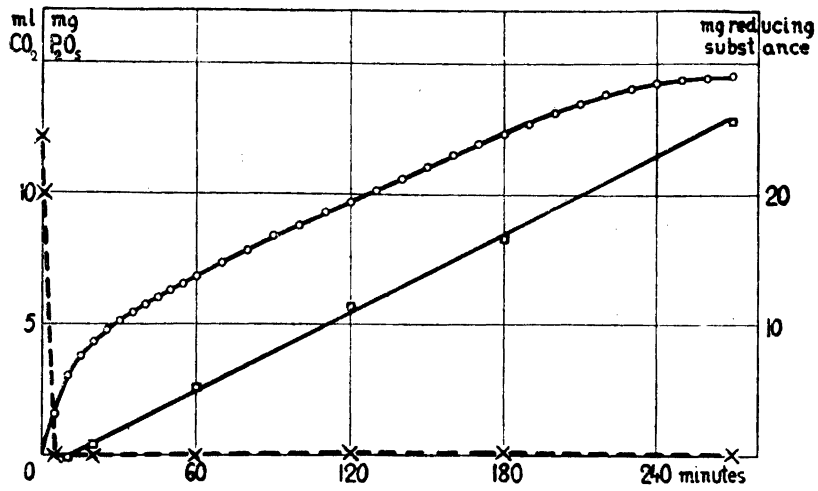


Fig. 10. Fermentation in maceration juice of 100 mg of glucose without addition of phosphate. Temp. 30° C. (Methods p. 220).

○ ——— ○ *Evolution of CO_2.*

× ——— × *Disappearance of inorganic phosphate.*

□ ——— □ *Formation of reducing substance.*

It appears from Fig. 9 and the accompanying Tables 5 and 6 that the amount of trehalose formed is augmented with increasing amounts of sugar. No quantitative agreement exists between the amount of trehalose formed and the sugar deficit calculated. The experiment nevertheless provides support for the view that the trehalose is formed from the sugar added and does not consist of trehalose that in one state or another is preformed in the maceration juice.

The formation of trehalose during the fermentation process is illustrated by Fig. 10.

As will be seen from Fig. 10, the formation of trehalose sets in only after the first break in the fermentation curve has been reached. The phosphate is then already practically completely esterified, and no splitting off of the phosphate, measurable with the analytic method employed here, takes place during the entire period of the experiment. The formation of trehalose proceeds along a practically straight line. This appears to us to remove to an essential degree the suspicion that the trehalose found might in some state be preformed in the maceration juice. If the maceration juice contained trehalose phosphate, the transphosphorylation of its phosphate to the glucose present in great excess in the fermenting mixture should not reasonably, purely from the kinetic point of view, proceed along a straight line.

Finally, we will return in this connection to the experiment reported in Fig. 1 (p. 215). As has already been pointed out (p. 216), added glucose produces with intact dried yeast only half of the calculated amount of CO_2 in the fermenting mixture there used (Curve C). Here we are dealing with an equimolar mixture of glucose and hexosediphosphate, and it is natural to assume that a transphosphorylation here takes place between the hexosediphosphate (or the triosephosphates in equilibrium with it) and the glucose added. It seems possible that the sugar deficit, which in this case amounts to half of the sugar added, consists of trehalose. The senior author has with M. Elander made preliminary experiments on the fermentation mechanism in such mixtures of sugar and esters. The present authors hope for the opportunity of resuming this investigation.

As has been mentioned above (p. 219) our earlier experiments do not sufficiently illustrate the mechanism at the earlier described production of trehalose. The following possibility should be closer investigated:

$$\text{hexosemonophosphate} + \text{glucose} \rightleftharpoons \text{trehalose} + \text{phosphate}$$

In this connection ought to be mentioned the reaction studied by Doudoroff [21]:

$$\text{glucose-1-phosphate} + \text{fructose} \rightleftharpoons \text{sucrose} + \text{phosphate}$$

With the phosphorylase used by Doudoroff could, however, no phosphorolysis of trehalose be received; nor could fructose be replaced by glucose in that system.

SUMMARY

1. Starting from our earlier investigations into the mechanism of the alcoholic fermentation in the living yeast cell, Meyerhof's researches on the rôle of adenylpyrophosphatase in fermentation are discussed. It is demonstrated that several of our observations are given no explanation through Meyerhof's investigations and cannot agree with the view that the zymase system of the living yeast cell differs from that of the maceration juice only by a higher content of adenylpyrophosphatase.

2. It is demonstrated that in fermentation in maceration juice with an excess of sugar versus phosphate a continuous formation of a low molecular, non-reducing substance takes place between the first and the second break in the fermentation curve. This substance has the optical rotation of trehalose, and on hydrolysis yields a product with the optical rotation of glucose, which with living yeast as well as with maceration juice is fermented in the

same way as glucose. It seems probable that this can provide an explanation of the »sugar deficit» established and discussed in our earlier publications, that is present at the second break. The quantitative conditions are not yet sufficiently clear, however, for it to be regarded as definitely proved that the sugar deficit is entirely covered by the trehalose formed.

3. The conditions prevailing in fermentation in mixtures of sugar and esters are discussed.

4. The mechanism of the formation of trehalose is discussed.

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