LXII. THE ENZYMATIC FORMATION OF POLYSACCHARIDES BY YEAST PREPARATIONS.

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Two reasons led to the institution of the following experiments. In an earlier paper [1904] the authors showed that the carbon dioxide evolved in the alcoholic fermentation of sugars by yeast juice was not equivalent to the sugar which disappeared from the solution, and ascribed this fact to the production of a hydrolysable compound of low reducing power. It was subsequently found that in alcoholic fermentation by yeast preparations a certain amount of hexosephosphate is formed, which has a lower reducing power than the sugar (about 75 per cent.) from which it is formed and would therefore in part account for the phenomenon.

In the second place it appears to follow from the authors' equations of fermentation [1908] that in the normal fermentation both of fructose and glucose half the sugar passes through the form of hexosephosphate, which is then hydrolysed. Since these two hexoses appear to yield the same hexosephosphate it would be expected that as the fermentation proceeded fructose and glucose alike would be partially converted into the same product of hydrolysis, and the rotations of their solutions should therefore tend to approximate to each other. The exact nature of the substance produced along with phosphoric acid by the hydrolysis of hexosephosphate in yeast juice is not definitely known, but all the evidence points towards its being fructose. Hence we should expect in all fermentations of glucose by yeast or yeast preparations a progressive conversion of glucose into fructose [compare Slator, 1911]. This however has not been observed and experiments were therefore made on the subject, especially with the object of ascertaining whether the product of hydrolysis of the hexosephosphate underwent any secondary change, such as condensation to a polysaccharide.

The present experiments show that both from glucose and fructose one or more dextrorotatory polysaccharides are produced during alcoholic

fermentation by yeast preparations. The previous conclusion of the authors is therefore confirmed, but it is not yet settled whether the polysaccharide formation takes place at the expense of the glucose and fructose themselves or occurs indirectly as the result of the action of some enzyme on the product of hydrolysis of the hexosephosphate. Further investigations on this point are in progress.

It is well known that living yeast forms glycogen when brought into excess of sugar solution [see Pavy and Bywaters, 1907], and the behaviour of yeast preparations therefore indicates that the enzymes involved in this synthesis are probably, at least to some extent, still present and active. The isolation of a substance having the qualitative reactions of glycogen is a further confirmation of the observation of Cremer [1899] who found that in yeast juice free from glycogen a substance was slowly formed in the presence of sugar which gave the characteristic glycogen reactions.

Experimental.

Experiment 1. Three lots of 100 cc. maceration juice (from Schroder's dried Münchener yeast) were incubated at 25° with toluene until they had attained the temperature of the bath. To Nos. 1 and 2 were then added 25 cc. of a 40 per cent. solution of glucose and the evolution of carbon dioxide observed. At the same time No. 3 was boiled and cooled, and 25 cc. of the same glucose solution added.

In Nos. 1 and 2 a maximum rate of 21·6 cc. per 2 minutes was slowly attained, which then rapidly diminished until in 52 minutes a constant rate of 2·8 cc. per 2 minutes was reached. The initial high rate was due to the presence of free phosphate in the maceration juice, which was converted into hexosephosphate. During this period the total gas evolved was 293·6 cc. at room temperature and pressure. After 52 minutes No. 2 was boiled, whilst the fermentation in No. 1 was allowed to proceed for 17 hours 38 minutes, during which time 1458 cc. of CO_2 had been evolved. No. 1 was then boiled. The contents of all three flasks were then filtered, and the free phosphate estimated in an aliquot portion of each. The amount of glucose in each was determined by precipitating the proteins in aliquot portions with Patein's mercuric nitrate solution and estimating the glucose by means of Pavy's method.

The treatment with mercuric nitrate precipitates the hexosephosphate, so that in order to determine the amount of sugar used up allowance must be made for the quantity bound up in the form of hexosephosphate. This is

readily done since it has given rise to the CO_2 equivalent to the free phosphate present at the beginning, and can therefore be determined by subtracting the carbonic acid corresponding with the constant rate of fermentation from the total actually evolved, in the manner frequently described before.

Free phosphate in No. 3 = 1·110 g., in No. 2 = 0·130 g. and in No. 1 = 0·127 g. $Mg_2P_2O_7$, showing that the same quantity of sugar is still bound up as hexosephosphate in No. 1 as in No. 2.

CO_2 evolved up to the time when No. 2 was boiled (after 52 mins. fermentation)	293·6 cc.
Rate = 2·8 × 26	72·8 ,,
Equivalent of phosphate	220·8 cc. or 201 cc. at N.T.P., i.e. 0·394 g.

This corresponds therefore to 0·79 g. glucose.

The amount of glucose converted into hexosephosphate may also be determined from the phosphate combined during the experiment. Phosphate bound up in No. 2 = 1·110 − 0·130 = 0·980 g. $Mg_2P_2O_7$; equivalent therefore to $0{\cdot}980 \times \frac{180}{222} = 0{\cdot}795$ g. glucose.

The tables show the amount of glucose which cannot be accounted for as CO_2 and alcohol or as hexosephosphate, the glucose originally present being obtained from No. 3 by analysis.

Flask (2).

CO_2 evolved = 293·6 cc. or 267 cc. at N.T.P. = 0·53 g. equivalent to 1·06 g. glucose.

Glucose bound up as hexosephosphate		0·79 g. ,,
Total accounted for		1·85 g. ,,
	Original glucose	9·98 g.
	Final glucose	8·15 g.
	Actual loss	1·83 g.

No disappearance of glucose was observed.

Flask (1).

CO_2 evolved = 1458 cc. or 1339 cc. at N.T.P. or 2·634 g. equivalent to 5·27 g. glucose.

Glucose bound up as hexosephosphate		0·79 g. ,,
Total accounted for		6·06 g. ,,
	Original glucose	9·98 g.
	Final glucose	0·91 g.
	Actual loss	9·07 g.

Thus 9·07 − 6·06 = 3·01 g. of glucose have disappeared.

The ratio between the reducing power and the optical rotation was determined in each mixture after the treatment with Patein's solution in

order to see if any active substance other than sugar were present. For the sake of convenience the rotation observed in a 400 mm. tube is compared with the reducing power determined by Pavy's method expressed as grams of glucose in 100 cc.

With pure glucose this ratio $\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} = +2{\cdot}05$, whilst with pure fructose it is $-4{\cdot}03$.

These ratios were found to be

$$(1) \quad +\frac{2{\cdot}149}{0{\cdot}120} = +17{\cdot}91.$$

$$(2) \quad +\frac{2{\cdot}224}{1{\cdot}08} = +2{\cdot}06.$$

$$(3) \quad +\frac{2{\cdot}843}{1{\cdot}32} = +2{\cdot}15.$$

It is thus seen that in No. 1 some substance is present which has a much greater dextrorotatory power than has glucose, whereas in No. 2 all the rotatory power may be accounted for by the quantity of glucose present.

Experiment 2. A similar experiment was carried out with fructose (Kahlbaum), the following mixtures being employed:

(1) 100 cc. maceration juice + 25 cc. 40 per cent. fructose + toluene.
(2) 100 cc. „ „ + 25 cc. 40 per cent. „ „
(3) 100 cc. „ „ + 25 cc. water + toluene.
(4) 100 cc. „ „ + 25 cc. „ „

The juice in each case was kept in the bath until the temperature was attained, and the fructose and water then added. Nos. 1 and 3 were boiled immediately, whilst the others were incubated and the fermentation observed. In No. 2, a high phosphate rate of 53 cc. per 5 minutes was rapidly reached which then decreased as usual to a constant rate of 7·5 cc. per 5 minutes; at the end of 17 hours both Nos. 2 and 4 were boiled. During the first 70 minutes No. 2 had given off 352·5 cc. of carbon dioxide, the amount due to the phosphate thus being $352{\cdot}5 - 14 \times 7{\cdot}5 = 352{\cdot}5 - 105 = 247{\cdot}5$ cc.; the total evolved in the 17 hours was 1179·5 cc. at 762·8 mm. and 15°. No. 4 showed no fermentation at all.

A portion of each of the four filtered mixtures was treated with Patein's solution as in the last experiment, and the reducing power and rotations determined in an aliquot portion of the filtrate. The figures given are calculated for the total volume of the juice.

	1. Original mixture	2. After 17 hrs.	3. Original juice alone	4. Juice incubated alone
Rotation in 400 mm. tube	−30·81°	+4·65°	−0·061°	−0·066°
Total sugar as g. glucose	9·31	1·10	0	0

Loss of sugar = (1) − (2) = 8·21 g.

CO_2 evolved = 1179·5 cc. at 762·8 mm. and 15° = 2·08 g.

CO_2 corresponding to fructose bound up as hexosephosphate = 247·5 cc. at 762·8 mm. and 15° = 0·41 g.

Total sugar accounted for as CO_2 and hexosephosphate therefore

$$= 2 \times 2{\cdot}49 = 4{\cdot}98 \text{ g.}$$

Sugar disappeared = 8·21 − 4·98 = 3·23 g.

The ratios of rotation to reduction expressed as before were also determined in the solution after treatment with Patein's solution and were found to be (1) − 4·14, (2) + 5·28; pure fructose = − 4·03.

It is thus seen that in this experiment a substance having a high dextrorotation was formed, so that although all the fructose was not used up, the mixture had changed in rotation from laevorotatory to dextrorotatory, whilst a much larger proportion of the original fructose had disappeared than could be attributed to the fermentation.

In the solutions before treatment with Patein's solution the free phosphate was estimated and was found to be:

(1) 1·203 g. $Mg_2P_2O_7$.
(2) 0·174 g. „
(3) 1·210 g. „
(4) 1·208 g. „

From this it is seen that the phosphate and hence an equivalent portion of the sugar was still bound up as hexosephosphate at the end of the experiment (No. 2). These numbers serve as before as a check on the quantity of sugar which has been converted to hexosephosphate, viz. that amount corresponding to

$$1{\cdot}203 - 0{\cdot}174 = 1{\cdot}029 \text{ g. } Mg_2P_2O_7 \text{ or } 1{\cdot}029 \times \tfrac{180}{222} \text{ fructose} = 0{\cdot}834.$$

Fructose calculated from equivalent of CO_2 as above = 0·41 × 2 = 0·82 g.

The mixtures before treatment with Patein's solution were tested with iodine solution; Nos. 1, 3, and 4 gave no colouration, whereas No. 2 gave a deep reddish brown colouration.

A portion of No. 2 treated with three volumes of alcohol gave a white precipitate, which was redissolved in water and again precipitated with alcohol. This last precipitate gave an opalescent solution in water which was precipitated by saturation with ammonium sulphate and gave a red colouration with iodine. The only difference which could be seen from the behaviour of glycogen was that it gave a somewhat different red colour with iodine.

The other solutions Nos. 1, 3, and 4 gave slight precipitates with alcohol, the aqueous solutions of which gave however no colouration with iodine.

It is thus seen that during the fermentation of fructose by maceration juice a dextrorotatory, glycogen-like substance is formed.

These results appear to us as already indicated to throw some light on the cause of the difference which exists between sugar fermented and carbon dioxide evolved, not only in the case of yeast preparations but also in that of living yeast. Euler and his colleagues in recent papers have argued from the existence of this difference between the amount of sugar actually removed by living yeast from a glucose solution and the amount equivalent to the CO_2 evolved, which he terms $\Delta - C$, that the hexose requires to undergo some change which renders it directly fermentable and that the difference $\Delta - C$ represents the amount which is in this intermediate condition. [Euler and Johannson, 1912; Euler and Berggren, 1912.] There seems however to be no good reason to suppose that Euler and Johannson's $\Delta - C$ cannot be accounted for by the well-known formation of glycogen which has been shown by Pavy and Bywaters [1907] to be of the order of magnitude required.

In Euler and Berggren's experiments on the effect of yeast extract in increasing both rate of fermentation and $\Delta - C$ [1912], no counts of yeast cells before and after the experiments were made. As the earliest observations were made after an hour at 15°–18° and the experiments in some cases extended to over six hours (1 g. pressed yeast in 25 cc. of solution), the possibility of yeast growth must not be overlooked. This is still more probable in the cases in which only 0·25 g. of pressed yeast was taken and tested with yeast extract itself, various precipitates from yeast extract, and with sodium nucleinate or ammonium formate [1912, pp. 216, 217; Euler and Cassel, 1913], in a total volume of 40 cc.

An experiment made on this point showed that under similar conditions of concentration growth readily occurs at 25°. The yeast was added as 5 cc. of a suspension of 5 g. yeast in 100 cc. H_2O, i.e. 0·25 g. yeast.

(1) and (2) 5 cc. yeast suspension + 20 cc. of 20 per cent. glucose + 15 cc. H_2O.

(3) and (4) 5 cc. yeast suspension + 20 cc. of 20 per cent. glucose + 15 cc. yeast extract.

In 345 mins. the evolutions were respectively 61, 59·6, 146·3, 150 cc. At the close of this time the numbers of cells present per cc. were $68{\cdot}7 \times 10^6$, $68{\cdot}5 \times 10^6$, $105{\cdot}25 \times 10^6$, $98{\cdot}8 \times 10^6$.

Asparagine acts in a precisely similar manner, 0·25 g. added to 0·5 g. yeast in 30 cc. sugar solution increasing the evolution in 2 hrs. at 25° from 73·6 to 89·4 cc.

It therefore seems that the experiments in which Euler has shown the accelerating effect of yeast extract, sodium nucleinate, etc., on the action of living yeast require revision from this point of view.

The method of testing for a co-enzyme by the action of solutions on living yeast is moreover open to the criticism that the yeast cell is, if at all, only imperfectly permeable to the co-enzyme so that negative results would be of little value.

SUMMARY.

During the alcoholic fermentation of glucose and fructose by Lebedeff's maceration extract of dried yeast, dextrorotatory polysaccharides are produced, and it is to the formation of these that the difference between the sugar removed and that equivalent to the carbon dioxide evolved is principally to be attributed.

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NOTE:

HASLAM. *Separation of proteins*, Part III, Globulins. This Journal 1913, **7**, 492.
In section 4 of Summary, p. 515, *for* 0·1 mg. P % *read* 0·1 P %.