CVII. THE FORMATION OF POLYSACCHARIDES BY YEAST PREPARATIONS.

By HIROSUKE NAGANISHI.

From the Biochemical Department, The Lister Institute, London.

(Received July 13th, 1926.)

It has been well known for a long time that a polysaccharide is synthesised from sugar by the enzymic action of micro-organisms. Cremer [1899] described how the fermentation of sugar by yeast-juice, which did not give a glycogen reaction with iodine, formed a new substance which gave a glycogen reaction. Harden and Young [1913] observed the production of dextrorotatory polysaccharides when glucose and fructose were fermented with Lebedev's maceration extract of dried yeast. In one case a substance was isolated, the solution of which was opalescent and gave a red coloration with iodine. Later Grey [1918] observed the formation of a non-reducing carbohydrate in the early stage of the fermentation of sugar by *B. coli* and he subsequently stated [1924] that starch giving the characteristic blue coloration with iodine was synthesised from sugar in the early stage of fermentation in the presence of chalk by an organism of the coli group.

Smedley-MacLean and Hoffert [1923] found that the presence of added phosphate slightly diminished the formation of polysaccharides in living yeast suspended in sugar solution, both in the presence and absence of oxygen.

The subject has acquired great interest owing to the recent investigations on the regeneration of glycogen in muscle, and the present work was undertaken in the hope (which was not fulfilled) of getting some knowledge of the mechanism of this change and particularly of the part, if any, played by phosphate in it. For this purpose experiments were made on the effect of addition of phosphate on the formation of polysaccharides from sugar by yeast-juice, maceration extract and zymin (yeast treated with acetone).

EXPERIMENTAL METHOD.

In order to ascertain whether polysaccharides had or had not been formed the following method was adopted. The sample under examination was diluted to a known bulk and at the same time sufficient *N* HCl was added to make the liquid *N*/10. In this way the fermentation was stopped and the precipitation of hexosephosphates in the next stage was prevented. In the case of zymin, the liquid was filtered at this stage through starch-free paper and the insoluble residue washed free of reducing sugar with *N*/10 HCl, hydrolysed for 4 hours in the water-bath with 0.6 *N* HCl and the reducing power (expressed

as glucose) determined by Bertrand's method after neutralisation and treatment with "dialysed iron."

To the original filtrate from the zymine, or, in the absence of zymine, to the diluted and acidified fermentation fluid, were then added 2 vols. of absolute alcohol, in order to precipitate glycogen, yeast-gum or any other polysaccharide which might be present. This precipitate was removed by centrifuging and washed with 66.6 % alcohol to remove sugar, etc. It was then dried over H_2SO_4 and in a weighed portion the amount soluble in water was determined as well as the reducing power and rotation of this soluble portion before and after hydrolysis for three hours with 0.6 *N* HCl. All the rotations refer to the mercury green line.

In the filtrate from the precipitate produced by 66.6 % alcohol the phosphates and hexosephosphates were precipitated by adding baryta until the liquid was faintly alkaline to phenolphthalein, sufficient alcohol being added at the same time to keep the alcohol concentration constant at 66.6 volumes %. (All concentrations of alcohol are expressed as cc. of alcohol in 100 cc. of the mixture.) A 5 % solution of barium acetate in 66.6 % alcohol was then added until no further precipitate was produced and the precipitate filtered off, thoroughly washed with 66.6 % alcohol, finally washed with absolute alcohol and dried. This precipitate was in some cases used for the estimation of the relative amounts of mono- and di-phosphoric esters formed and hence, indirectly, of the total glucose esterified. (See later.)

The final filtrate from the barium salts was evaporated at 45°, 0.5 *M* Na_2SO_4 was added to remove the barium and it was then diluted to 100 cc. and filtered. The filtrate, which contained any unfermented sugar, was then examined for reducing power and rotation before and after hydrolysis with HCl.

The precipitate of barium salts was examined, when necessary, by decomposing a weighed portion of it with dilute sulphuric acid and in the filtrate determining

(a) the reducing power, by Bertrand's method;

(b) the free and total phosphorus (Neumann), and hence by difference the combined phosphorus.

The amounts of hexosemonophosphoric acid and hexosediphosphoric acid present, and from these their glucose equivalent were then calculated by the simultaneous equation shown below.

$$\text{g. of combined phosphorus} = \frac{X \times 31}{260} + \frac{Y \times 62}{340} \dots\dots\dots(1).$$

$$\text{Direct reducing power (as g. of glucose)} = \frac{X \times 128}{260} + \frac{Y \times 72}{340} \dots\dots\dots(2).$$

X = g. of hexosemonophosphoric acid (Mol. wt. 260).

Y = g. of hexosediphosphoric acid (Mol. wt. 340).

This equation is based on the facts that the molecular reducing powers of the mono- and di-acids correspond respectively with those of 128 and 72 g. of glucose. The results are only approximate.

EXPERIMENTS AND RESULTS.

Exps. 1 and 2. In a first experiment with zymín, in which fermentation was only continued for 2 hours, it was found that there was scarcely any production of a polysaccharide. A second similar experiment was therefore made in which the fermentation liquids were examined after 12 and 24 hours. The experimental details are given in Table I. The phosphate and water were added after incubation for 1 hour.

Table I. (*Exp. 2.*)

No. of flask	Weight of zymín (g.)	Volume of 10 % glucose solution (cc.)	Volume of toluene (cc.)	Volume of 0.6 M phosphate solution (cc.)	Volume of water (cc.)	Time of fermentation (hours)	
1	2	15	0.2	—	—	1	
2	2	15	0.2	2.5	—	After addition 12	
3	2	15	0.2	—	2.5	„ 12	
4	2	15	0.2	2.5	—	„ 24	
5	2	15	0.2	—	2.5	„ 24	
Totals in 100 cc.							
			1	2	3	4	5
1. Glucose converted into CO ₂ , g.			0.043	0.821	0.397	0.948	0.594
2. Residue.							
Reducing power after hydrolysis (as g. glucose)			0.528	0.678	0.636	0.496	0.656
3. Precipitate by 66.6 % alcohol, g.			0.016	0.337	0.222	0.345	0.242
Rotation in 1 dm. tube			0	-0.13°	-0.30°	-0.13°	-0.34°
Reducing power, as g. glucose:							
(a) before hydrolysis			0	0	0	0	0
(b) after hydrolysis			0	0.023	0.014	0.019	0.012
4. Baryta precipitate.							
g. glucose combined as:							
(a) hexosemonophosphate			0.0002	0.008	0.0003	0.002	0.0004
(b) hexosediphosphate			0.036	0.156	0.062	0.115	0.069
5. Final filtrate.							
Reducing power, as g. glucose:							
(a) before hydrolysis			1.372	0.029	0.645	0	0.224
(b) after hydrolysis			1.396	0.132	0.713	0.116	0.297
Increase on hydrolysis			0.024	0.103	0.068	0.116	0.073

In this case there is evidence of production of a considerable proportion of polysaccharide, which is present in the final filtrate. The absolute quantity produced is greater in the two experiments in which phosphate was added (0.079 in 12 hours and 0.092 in 24 hours) than in the controls without added phosphate (0.044 in 12 hours and 0.049 in 24 hours), but it must be remembered that the amount of glucose converted into CO₂ was also greater in the former cases.

The ratios of the glucose converted into polysaccharide to the glucose fermented to CO₂ and to that converted into hexosephosphate were as follows:

	Experiment			
	2 12 hours Phosphate added	3 12 hours No phos- phate added	4 24 hours Phosphate added	5 24 hours No phos- phate added
Glucose to polysaccharide Glucose fermented to CO ₂	0.100	0.101	0.121	0.086
Glucose to polysaccharide Glucose converted into hexosephosphates	0.609	1.68	1.14	1.43

It appears that the amount of glucose converted into this polysaccharide is roughly 10 % of the glucose fermented, but bears no definite relation to the amount of hexosephosphates produced. Only traces of a polysaccharide precipitated by 66.6 % alcohol from *N*/10 HCl solution were observed. It was, however, noticed that in Nos. 2, 3 and 5 the residue gave on hydrolysis a higher reducing power than the control. The cause of this was not ascertained.

Exp. 3. In order to obtain further information as to the nature of this polysaccharide an experiment on a somewhat larger scale was made. 50 g. of zymin, 37.5 g. glucose and 62.5 cc. of 0.6 *M* phosphate being used in a total volume of 437.5 cc. and incubated for 12 hours (Table II).

 Table II. (*Exp. 3.*)

	Totals in 500 cc.	
	1 Phosphate added	2 H ₂ O added
<i>Final filtrate.</i>		
Reducing power as g. glucose		
(a) before hydrolysis	1.271	14.629
(b) after hydrolysis	4.584	17.237
Increase on hydrolysis	3.313	2.608
Rotation in 1 dm. tube:		
(a) before hydrolysis	+1.16	+2.19
(b) after hydrolysis	+0.67	+1.73

As before, no glycogen was detected in the precipitate produced by 66.6 % alcohol, but yeast-gum was present and was detected by the formation of a bulky gelatinous precipitate with Fehling's solution.

The final filtrate, after removal of hexosephosphates and yeast-gum, contained a considerable amount of the polysaccharide as shown by the change in reduction (calculated on the whole amount) and rotation on hydrolysis with acid.

These two final filtrates were fractionated by precipitation with alcohol but no sharp separation of the polysaccharide was obtained. Thus in No. 1, in which the residual sugar (as measured by reducing power) was comparatively small (0.25 %), after the removal of a small amount of non-saccharine material (*A*) by 75 % alcohol, fractions containing polysaccharide were precipitated from the five times concentrated filtrate by 80 % (*B*) and by 95 % alcohol (*C*) and some of it was still present in the filtrate (*D*).

	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>d</i>
Concentration of alcohol (% by volume)	75	80	95	—	95
Increase in reduction on hydrolysis (as g. of glucose)	0	0.412	0.082	0.526	0.494
Decrease in rotation in 1 dm. tube on hydrolysis (total volume 50 cc.)	0	0.445	0.04	0.67	0.72

In No. 2 (no phosphate added) the residual sugar concentration was much higher (4.15 %) and the results still less sharp. The purest product obtained

(d) closely resembled those obtained in the presence of added phosphate. This was precipitated from 24 cc. of a ten times concentrated solution by addition of 19 volumes of alcohol. It was then dissolved in 40 cc. of water, treated with 9 volumes of alcohol, filtered, evaporated to 8 cc. and precipitated by 19 volumes of alcohol. None of these precipitates gave a coloration with iodine or a precipitate with Fehling's solution.

Exps. 4 and 5. Experiments were next made in a similar manner with yeast-juice prepared by grinding top yeast (Table III).

Table III. (*Exp. 4.*)

No. of flask	Volume of yeast-juice (cc.)	Weight of glucose (g.)	Volume of toluene (cc.)	Volume of 0.6 M phosphate solution (cc.)	Volume of water (cc.)	Volume of 4 N HCl (cc.)	Time of fermentation (hours)
1	25	5	0.2	10	—	10	0.5
2	25	5	0.2	10	—	10	17.0
3	25	5	0.2	—	—	—	0
4	25	5	0.2	10	—	—	17.0
5	25	5	0.2	—	10	—	17.0
Totals in 100 cc.							
				3	4	5	
1. Glucose converted into CO ₂ , g.				0	1.354	0.537	
2. Precipitate by 66.6 % alcohol.							
Reducing power as g. glucose:							
(a) before hydrolysis				0.011	0.009	0	
(b) after hydrolysis				0.167	0.058	0.065	
Increase on hydrolysis				0.156	0.049	0.065	
Iodine reaction				+	—	—	
3. Baryta precipitate.							
g. glucose combined as:							
(a) hexosemonophosphate				0.083	0.305	0.061	
(b) hexosediphosphate				0.100	0.412	0.122	
4. Final filtrate.							
Reducing power as g. glucose:							
(a) before hydrolysis				4.628	2.224	3.777	
(b) after hydrolysis				4.831	2.712	4.249	
Increase on hydrolysis				0.203	0.488	0.472	
Rotation in 1 dm. tube:							
(a) before hydrolysis				+3.00°	+2.09°	+3.05°	
(b) after hydrolysis				+2.69°	+1.43°	+2.49°	
5. Total glucose accounted for, g.				5.181	4.841	5.034	
Glucose not accounted for, g.				—	0.34	0.147	

An attempt was made in this case to account for the whole of the carbohydrate, both that originally present in the yeast-juice and that added as glucose. Actually, discrepancies of 6.6 % and 2.9 % were found. In order to obtain a more accurate estimate of the CO₂ produced by fermentation, the combined CO₂ present in the liquid before and after fermentation was estimated in parallel samples by adding excess of hydrochloric acid (saturated with CO₂), measuring the volumes evolved, and correcting for any difference in the values obtained before and after fermentation.

ENZYMIC FORMATION OF POLYSACCHARIDES BY YEAST 861

The original juice contained a certain amount of glycogen, for the precipitate produced in the acid solution by 2 volumes of alcohol gave the iodine reaction for this substance. This disappeared during fermentation and no glycogen was detected in either of the fermented samples. It also contained a considerable amount of polysaccharide soluble in 66.6 % alcohol, as the final filtrate gave an increase of reducing power (equivalent to 0.203 g. glucose) and a decrease of rotation (0.31° in 1 dm. tube) on hydrolysis.

The total polysaccharides of the original juice (expressed as g. of glucose) and of the two fermented juices were as follows:

	3	4	5
Precipitated by 66.6 % alcohol	0.158	0.048	0.065
In final filtrate	0.203	0.488	0.472
	0.361	0.536	0.537

There was, therefore, an increase on fermentation corresponding at least to 0.167 g. glucose and this increase was in this case the same in the presence and absence of added phosphate, although the amount of CO_2 evolved in the absence of added phosphate (0.54 g.) was much less than in its presence (1.354 g.).

Table IV. (*Exp. 5.*)

No. of flask	Volume of yeast-juice (cc.)	Weight of glucose (g.)	Volume of toluene (cc.)	Volume of 0.6 M phosphate solution (cc.)	Volume of water (cc.)	Duration of fermentation (hours)
1	230	46	2	92	0	17
2	230	46	2	0	92	17

(No. 1) I. 230 cc. of final filtrate + 4 volumes of absolute alcohol.
Precipitate A; dissolved in 100 cc. of water.
II. Filtrate from I evaporated down to 100 cc. at 45° + 9 volumes of alcohol.
III. Precipitate from II dissolved in 50 cc. of water + 9 volumes of absolute alcohol.
IV. Precipitate from III dissolved in 20 cc. of water + 3 volumes of absolute alcohol.
Precipitate B; dissolved in 50 cc. of water.
V. Filtrate from IV + 1.5 volumes of absolute alcohol.
Precipitate C; dissolved in 50 cc. of water.

The final filtrate of No. 2 was treated in a similar manner and yielded corresponding fractions, a, b and c.

	No. 1			No. 2		
	A in 100 cc.	B in 50 cc.	C in 50 cc.	a in 100 cc.	b in 50 cc.	c in 50 cc.
Reducing power (as glucose):						
(a) before hydrolysis	0.144	1.075	0.304	0.096	0.626	0.409
(b) after hydrolysis	0.371	1.973	0.562	0.328	1.359	0.702
Increase on hydrolysis	0.227	0.898	0.258	0.232	0.733	0.293
Rotation in 1 dm. tube:						
(a) before hydrolysis	+0.66°	+4.23	+1.11	+0.33	+3.02	+1.32
(b) after hydrolysis	+0.22	+2.35	+0.64	+0.19	+1.60	+0.74

Parallel with the foregoing a larger scale experiment (*Exp. 5*) was carried out with two quantities of 230 cc. of the same preparation of yeast-juice + 45 g. of glucose, to one of which (1) 92 cc. of 0.6 M phosphate were added and to the

other (2) 92 cc. H_2O . The final filtrates were then fractionated with alcohol and the resulting precipitates (see Table IV) examined. As before, a considerable amount of polysaccharide was found in each of these. As *B* and *b* contained the largest quantity these were submitted to refractionation by 80 and 90 % alcohol and thus divided into three fractions, which were then examined (Table V).

Table V. (*Exp.* 5.)

	Totals in 50 cc.					
	X	Y	Z	<i>x</i>	<i>y</i>	<i>z</i>
	Pre- cipitated by 80 %	Pre- cipitated by 90 %	Soluble in 90 %	Pre- cipitated by 80 %	Pre- cipitated by 90 %	Soluble in 90 %
Reducing power as g. glucose:						
(a) before hydrolysis	0.278	0.117	0.580	0.165	0.094	0.476
(b) after hydrolysis	0.762	0.262	0.803	0.462	0.187	0.650
Increase on hydrolysis	0.484	0.145	0.223	0.297	0.093	0.174
Rotation in 1 dm. tube:						
(a) before hydrolysis	+2.05°	+0.67°	+1.47°	+1.29°	+0.47°	1.18°
(b) after hydrolysis	+0.92°	+0.29°	+0.90°	+0.45°	+0.15°	0.60°

The purest of these fractions was probably *X*, and, if it can be assumed that it was free from glucose, its rotatory and reducing powers, expressed in terms of those of the glucose formed from it by hydrolysis, are rotation 2.16, and reduction 0.36; *x* possessed almost the same characteristics; rotation 2.26, reduction 0.36.

Exp. 6. Another large-scale experiment with 500 cc. of yeast-juice gave very similar results (Table VI). The final filtrate from the original yeast-juice was found to contain a considerable amount of the polysaccharide, which had precisely similar properties to that isolated after fermentation and behaved in the same manner on fractionation. This amount diminished when the yeast-juice was incubated for 17 hours in the absence of added sugar, but increased in the sample to which sugar and phosphate were added before incubation.

In this case the original yeast-juice and the liquid after fermentation with glucose and phosphate for 17 hours contained practically the same amount of glycogen, whilst the sample which was incubated without sugar for 17 hours had lost its glycogen. Whether this indicated merely a delayed hydrolysis of the original glycogen in presence of glucose and phosphate or an actual new formation could not be decided.

Exp. 7. Finally, to complete the series, an experiment was made with maceration extract prepared by Lebedev's method from a dried mild ale top yeast (Table VII). This contained very little polysaccharide either in the precipitate produced by 66.6 % alcohol or in the final filtrate and also contained practically no hexosephosphate. Very little polysaccharide was found in the final filtrate after incubation with glucose alone or in presence of excess of phosphate (10 cc. of 0.6 *M* to 25 cc. juice), although a moderately vigorous

ENZYMIC FORMATION OF POLYSACCHARIDES BY YEAST 863

evolution of CO₂ occurred. In experiments in which only 5 cc. of the phosphate solution were added and in which a much more vigorous fermentation occurred, there was a decided formation of the polysaccharide, both from glucose and fructose. The products were not further investigated.

No glycogen was produced in this experiment.

Table VI. (*Exp. 6.*)

No. of flask	Yeast juice (cc.)	Glucose (g.)	Toluene (cc.)	0.6 M phosphate (cc.)	Duration of fermentation (hours)
1	500	0	0	0	0
2	500	50	5	200	17
3	100	0	1	0	17
Calculated for 100 cc. yeast juice.					
<i>Final filtrate.</i>		1	2	3	
Reducing power as g. glucose:					
(a) before hydrolysis		0.017	0.618	0.041	
(b) after hydrolysis		0.619	1.401	0.605	
Increase on hydrolysis		0.602	0.783	0.564	
Fractionation by alcohol of final filtrates 1 and 2. $\frac{1}{10}$ of each filtrate (after removal of alcohol) was used for this purpose.					
1. 80 % precipitate. 400 cc. evaporated to 200 cc. and 4 volumes of alcohol added. The precipitate was dissolved in 60 cc. of water and precipitated by 4 volumes of alcohol, this process being repeated twice.					
2. 85 % precipitate. The filtrate from 1 was evaporated to 80 cc. and made up to 85 % with alcohol. The precipitate was then dissolved up and reprecipitated with 85 % alcohol and this process repeated twice.					
3. 90 % precipitate. The filtrate from 2 was made up to 90 % by the addition of alcohol and the precipitate redissolved in 30 cc. and reprecipitated by 90 % alcohol.					
4. Residue. The filtrate from 3 was evaporated to 100 cc.					
Examination of the fractions obtained from final filtrates 1 and 2.					
(Each purified fraction was made up to 100 cc. with water.)					

Rotation (1 dm.)		1	2
80 % precipitate	(a) before hydrolysis	+0.56°	+1.65°
	(b) after "	+0.20	+0.52
85 % "	(a) before "	+0.68	+0.74
	(b) after "	+0.22	+0.26
90 % "	(a) before "	+0.09	+0.12
	(b) after "	+0.02	+0.04
Residue	(a) before "	-0.50	-0.63
	(b) after "	-0.26	-0.30
Reducing power (as g. glucose)			
80 % precipitate	(a) before hydrolysis	0.010	0.086
	(b) after "	0.348	0.917
85 % "	(a) before "	0.083	0.070
	(b) after "	0.423	0.423
90 % "	(a) before "	0	0
	(b) after "	0.054	0.073
Residue	(a) before "	0.061	1.646
	(b) after "	0.115	1.885

CONCLUSIONS.

The foregoing experiments afford abundant confirmation of the observation made by Harden and Young of the enzymic production of polysaccharides in yeast-juice and similar preparations. Substances of a similar character are present in freshly prepared yeast-juice in addition to glycogen and yeast-gum.

Table VII. *Exp. 7. 1. xii. 1925.*(Maceration juice from dried mild ale yeast. 14. iv. 1925)
(With 0.2 cc. toluene in each experiment)

Date	Exp. no.	Maceration juice (cc.)	Glucose (g.)	0.6 M phosphate mixture (cc.)	Water (cc.)	Duration of fermentation (hours)		
1. xii. 1925	1	25	0	0	0	0		
"	2	25	5	0	0	0		
"	3	25	0	0	0	17		
"	4	25	5	0	10	17		
"	5	25	5	10	0	17		
3. xii. 1925	6	25	5	5	0	17		
5. xii. 1925	7	25	2.5	5	0	18		
"	8	25	2.5*	5	0	18		
* Fructose (B.D.H.).								
	1	2	3	4	5	6	7	8
1 Glucose converted into CO ₂	0	0	0	0.677	0.464	—	—	1.276
2 Precipitated by 66.6 % alcohol.								
Reducing power as g. glucose:								
(a) before hydrolysis	0.004	0.015	0.003	0.027	0.004	0.020	0.010	0.007
(b) after hydrolysis	0.054	0.135	0.095	0.203	0.106	0.195	0.120	0.109
Iodine reaction for glycogen	—	—	—	—	—	—	—	—
3. <i>Baryta precipitate.</i>								
g. glucose combined as:								
(a) hexosemonophosphate	0	0	0	0.667	0.368	0.034	0.174	0.258
(b) hexosediphosphate	0	0	0	0.002	0.119	1.083	0.753	0.180
4. <i>Final filtrate.</i>								
Reducing power as g. glucose:								
(a) before hydrolysis	0	4.859	0	3.243	3.712	2.041	0.008	0.002
(b) after hydrolysis	0	4.838	0	3.238	3.735	2.174	0.130	0.179
Increase on hydrolysis	0	-0.021	0	-0.005	0.023	0.133	0.122	0.177
Rotation in 1 dm. tube (total volume 100 cc.):								
(a) before hydrolysis	-0.17°	+2.75°	-0.23°	+1.75°	+2.30°	+1.34°	+0.05°	+0.575°
(b) after hydrolysis	-0.14°	+2.80°	-0.18°	+1.78°	+2.20°	+1.15°	-0.04°	+0.140°

The formation of these substances was found to be irregular and no definite conditions were found which specially favoured it. In particular no relation was found between the amount of phosphoric esters formed and the degree of polysaccharide formation. No definite evidence of the production of glycogen was obtained in these experiments.

Among the compounds formed are substances which are probably dextrans, possessing about twice the rotatory power and about 36 % of the reducing power of the glucose formed from them by hydrolysis¹.

The investigation is being continued.

I desire to express my thanks to Prof. A. Harden for the valuable advice and assistance he has given me in the prosecution of this investigation.

REFERENCES.

- Cremer (1899). *Ber. deutsch. chem. Ges.* **32**, 2062.
 Grey (1918). *Proc. Roy. Soc. Lond. B*, **90**, 75, 92.
 — (1924). *Biochem. J.* **18**, 712.
 Harden and Young (1913). *Biochem. J.* **7**, 630.
 Smedley-MacLean and Hoffert (1923). *Biochem. J.* **17**, 720.

¹ All the products obtained contained a certain amount of mineral matter.