CCXII. YEAST FERMENTATION OF PENTOSE PHOSPHORIC ACIDS

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IN all references to pentoses in textbooks on sugars, the fact that they are not fermented by yeast [Salkowski, 1900] is prominent. In the following it is shown that while the pentoses are not fermented appreciably either by yeast cells or by Lebedew extract, phosphorylated pentoses are fermented, and that in the case of the *d*-ribose-5-phosphoric ester, the fermentation by yeast extract is about as rapid as that of glucose or hexosemonophosphate. The fermentation of *d*-ribose-5-phosphoric acid requires the presence of cozymase, yeast enzyme and inorganic phosphate, and probably of metal salts (Mg and Mn) also. (Adenylic acid was present in the cozymase used.) The products of fermentation are per mol. ribosephosphate, 1 mol. CO₂, 1 mol. alcohol and 1 mol. inorganic phosphate. In addition an unknown, HIO₄-reducing substance appears, which may perhaps be glycol.

The natural pentose phosphoric acid of animal and yeast cells—*d*-ribose-5-phosphoric acid—is fermented far more rapidly than either *d*-arabinose-5-phosphoric acid or the corresponding xylose compound. This makes it possible that *d*-ribose-5-phosphoric acid may play an important part as an intermediate both in aerobic fermentation and in sugar oxidation [Dickens, 1938]. These facts may eventually prove of significance in connexion with the mechanism of the Pasteur reaction and in carbohydrate metabolism generally. The part played by pentose phosphoric acids in metabolism is being further studied.

Methods

Alcohol estimation. The method of Friedemann & Klass [1936] was used and proved satisfactory provided that all the precautions described were rigidly observed. Erratic results are obtained unless all glassware is cleaned with chromic-sulphuric acid immediately before use. The distillation apparatus was modified to suit existing equipment, and with the glass condenser used the recovery was as follows: μ l. alc. taken (1 mM. = 22400 μ l.); 224, 224, 45, 45; recovered; 90, 91, 93.5, 88 % mean recovery 90.5 %. Redistillation from Ca(OH)₂-HgSO₄ was always used after the distillation from HgSO₄, to ensure removal of interfering substances. Pentose phosphoric esters did not give any volatile titratable material. It is essential to have a large excess of KMnO₄ during oxidation.

Reducing substances. The Cu reduction method of Somogyi [1937] was adapted for the separate estimation of pentose and pentose phosphoric acid. The reducing values of *d*-ribose, *d*-ribose-5-phosphoric acid and glucose found for our preparations were respectively 58 %, 57 % and 100 % for equimolecular quantities (ribose-phosph. calc. on P content of soln. of Na salt). Neutral Pb acetate was found to remove ribosephosphate practically quantitatively from its solution and the loss of free ribose under the same conditions was negligible.

Method. The proteins are removed with tungstic acid as usual. The perfectly clear centrifugate is pipetted into a dry centrifuge tube, an aliquot being used for total reducing substance. The ribosephosphate is precipitated by neutral Pb acetate, allowing to stand for $\frac{1}{2}$ hr. Centrifuging removes the lead salt and the clear or faintly opalescent supernatant fluid is pipetted into a dry centrifuge tube, excess $M/2$ Na_2HPO_4 added, Pb phosphate removed by centrifuging and a measured amount of the perfectly clear supernatant fluid used for the estimation of reducing substance not precipitable by neutral Pb acetate. 0.03 ml. $M/10$ solution of ribosephosphate per ml. tungstate filtrate was the concentration usually employed for the Pb precipitation.

HIO₄ titration. This was used for detection and estimation of substances containing two or more adjacent free OH groups (micro-method modified from Fleury & Fatome [1935]). It was found that 1 ml. $M/50$ soln. of each of the following required the approximate amounts of $N/50$ I_2 stated: glycerol, 4.0; glucose, 8.8; ribose, 6.4; hexosemonophosphate, 7.1; ribosemonophosphate, 5.3; glycol, 2.0 ml. The method is applicable to the Na_2HPO_4 supernatant fluid mentioned above, provided that it is acidified to litmus before adding the HIO_4 . Quantities below 1 ml. $M/100$ can be estimated with accuracy, but the method is only applicable if sugars and interfering substances are known to be absent.

Materials

Pentose-5-phosphoric acids. The natural *d*-ribose-5-phosphoric acid and synthetic *d*-arabinose- and xylose-5-phosphoric acids (Levene) used have already been described [Dickens, 1938], together with the prep. of the $M/10$ soln. of the Na salts. *d*-Ribose was made from guanosine (B.D.H.) according to Levene & Clark [1921]. It crystallized readily in the desiccator.

Cozymase was a purified prep. made in this laboratory by Dr Weil-Malherbe.

Muscle adenylic acid was obtained from Fraenkel and Landau. It appeared to contain cozymase, and as the cozymase contained adenylic acid, adenylic acid was not added in more than a few exp.

Mg-Mn soln. 0.025 M MgSO_4 containing 0.005 M MnSO_4 .

Ca hexosediphosphate B.D.H. prep. *Mg hexosediphosphate* kindly presented by Prof. Schoeller, Schering-Kahlbaum. $M/50$ soln. was used, the Ca salt being dissolved by partial decomp. by Na_2SO_4 .

Glycollaldehyde was prepared from dihydroxymaleic acid by distillation after heating with pyridine [Fischer & Taube, 1927]: it crystallized readily after distillation.

Enzyme preparations. Lebedew fluid was prepared from Löwenbräu, Munich, bottom yeast. The dialysed, dried preparation already described [Dickens, 1938] was also made from this yeast.

Fermentation of pentose and pentosephosphoric acids by Lebedew fluid and by yeast cells

Yeast cells. D.C.L. Company's yeast was washed three times at the centrifuge with dist. water and a 2% suspension in $M/25$ KH_2PO_4 prepared. 1 ml. per vessel was transferred to Warburg manometers and the substrates were added to make the conc. $M/15$. Fermentation was measured in N_2 at 37.5° (Fig. 1). Ribose was not at all fermented, ribosephosph. was slightly attacked, but the action was very small compared with the very vigorous fermentation of glucose and may have been due to a trace of impurity, but in such cases there is usually a sharp fall of reaction rate, which was not so here.

Lebedew fluid. Fig. 2 shows that contrary to what happens with the intact cells, fermentation with ribose phosph. is very vigorous in Lebedew extract, and ribose is also attacked, though relatively very much more slowly.

Products and end-point of fermentation of ribosephosphate by Lebedew fluid. Fig. 3 shows the course of the reaction. The end-point of fermentation of 9 ml.

Lebedew fluid with 2.4 ml. 0.76 *M*/10 ribosephosph. was 7840 μ l. CO₂; blank (9 ml. + 2.4 ml. water) 735 μ l. CO₂. Diff. 7105 μ l. CO₂. Ribose P taken = 8170 μ l. (22400 μ l. = 1 ml. *M* soln.). Ratio: mol. CO₂/mol. ribose P = 0.86.

Fig. 1.

Fig. 2.

Fig. 1. Yeast cells. Fermentation of glucose, ribose and ribosephosphate. Conc. *M*/15.

Fig. 2. Fermentation with Lebedew macerate (1 ml.). Conc. of substrates in mixture are shown.

Fig. 3.

Fig. 4.

Fig. 3. Fermentation of ribosephosphate by Lebedew fluid: end-point, alcohol and P estimations.

Fig. 4. Fermentation of various substrates by Lebedew fluid (1 ml.): ml. *M*/10 substrates added as shown.

Increase of inorganic P in trichloroacetic acid filtrate = 6950 μ l. = 98 % of CO₂ evolved and = 85 % of ester P present in the ribosephosph. Alcohol increase, 6300 μ l. = 90 % of CO₂ formed (1 m.Mol. H₃PO₄ or C₂H₅OH = 22,400 μ l.)

This exp. shows that ribosephosph. is decomposed to the extent of 86 % by Lebedew fluid; that CO₂ equivalent to 85 % of 1 mol. CO₂ per mol. total ribosephosph. is liberated; that the alcohol formed, assumed to be ethyl alcohol, is 77 % of the total ribose ester taken, and that inorganic phosphate is split off equivalent to the CO₂ formed.

Fermentation of other pentose phosphoric acids by Lebedew fluid

Fig. 4 shows the result of incubating various substrates with Lebedew fluid. Arabinosephosph. and xylosephosph. are only slightly fermented. With ribosephosph. we have seen that only 1 mol. CO₂ is liberated per mol. substrate, hence to compare the rate of reaction with those of hexosemonophosphate and glucose, which give 2 mol. CO₂ per mol., half quantities of these substrates were used (Fig. 4). When this is done, the rate of fermentation of ribosephosph. is close to that of glucose, and about equal to that of hexosemonophosphate (Robison ester).

In view of the fact that the pentoses themselves are practically non-fermentable, this observation opens a new field for investigation.

The alcohol estimations in the exp. of Fig. 4 were as follows:

Table I. *Alcohol estimations and CO₂ liberation*

Substrate	Water	0.3 ml. 0.05 M glucose	0.15 ml.	0.3 ml.	0.3 ml.	0.3 ml.	0.15 ml.
			0.091 M hex. mono- phosph.	0.076 M ribose phosph.	0.08 M arab. phosph.	0.08 M xyl. phosph.	0.076 M rib. phosph.
μ l. substrate	0	336	303	511	538	538	256
μ l. CO ₂ evolved	64	656	514	625	75	115	298
Increase CO ₂ over blank	0	592	450	561	11	51	234
Calc. CO ₂ for mols.	—	2	2	1	1	1	1
" μ l.	—	672	606	511	538	538	256
($\frac{\text{Found}}{\text{Calc.}}$) CO ₂ %	—	88	74	110	2	9.5	91
μ l. alcohol (increase over blank)	—	732	—	582	—	—	—

With Lebedew fluid, the alcohol increase, being small in proportion to the relatively high alcohol content of the macerate, can only be measured approximately. The CO₂ retention of Lebedew fluid, despite its acid reaction, is also appreciable. These objections apply less to the enzyme experiments.

Fermentation of ribosephosphate by dried enzyme

Each Warburg vessel contained 80 mg. dried dialysed Lebedew extract dissolved in 1 ml. water and centrifuged. 0.1 ml. Mg-Mn soln., M/2 KH₂PO₄, M/50 hexose-diphosph., adenylic acid (1 mg./ml.), and cozymase (2 mg./ml.), and 0.2 ml. acetaldehyde (25 mg./ml.) and glycollaldehyde (25 mg./ml.) were added as required. In all exp. unless otherwise stated 0.3 ml. M/10 ribosephosphate and 0.3 or 0.15 ml. M/10 hexosemonophosphate were the substrates used. N₂. 37.5°.

The results are shown in Figs. 5-7.

Induction period. Fig. 5 shows that fermentation with the dried enzyme sets in only after the addition of an activator (in one exp. a spontaneous onset of fermentation after 30 min. was observed; this may have been due to accidental

contamination with some activating substance). In the exp. of Fig. 5 the actions of acetaldehyde with ribosephosphate and hexosemonophosphate are compared.

Fig. 5.

Fig. 6.

Fig. 5. Dried enzyme: activation of fermentation of ribosephosph. by acetaldehyde.

Fig. 6. Dried enzyme: comparison of activators (acetald. $M/20$, glycollaldehyde $M/30$, hexose-diphosphate $M/900$).

Fig. 7.

Fig. 8.

Fig. 7. Dried enzyme: need for coenzyme I. 0.3 ml. ribosephosph., 0.15 ml. hexosemonophosph.

Fig. 8. Dried enzyme: need for inorg. P; effect of addition of Mg and Mn.

Fig. 6 gives a comparison of the activation by acetaldehyde with that of hexosediphosphate and glycollaldehyde. Glycollaldehyde was tested because it has often been suggested as a breakdown product of pentoses and was described by Neuberg & Sandberg [1921] as an exceptionally vigorous activator in hexose fermentation. It was less efficient than the other two substances, of which hexosediphosphate was the better. (0.1 ml. *M*/50 hex. diphosph. = 45 μ l. per vessel.) The action resembles that well known in hexose fermentation. This amount of hexosediphosphate was added in all the following experiments.

Coenzyme. Fig. 7 shows that cozymase is necessary for fermentation to proceed, and also shows the end-point of the fermentation clearly. Many similar results were obtained. The cozymase used was that tested previously [Dickens, 1938] and shown to be free from coenzyme II; it probably contained adenylic acid.

Phosphate and mineral salts. Fig. 8 shows that inorganic phosphates are a necessary component for fermentation to occur. (Dialysed enzyme free from inorg. P; other reagents also, except ribosephosph. which contained 0.06 mg. (40 μ l.) inorg. P in the 0.3 ml. used.) Addition of 112 μ l. inorg. P produced full activation, smaller quantities were not tested. By analogy with hexose fermentation this suggests the primary formation of a pentosediphosphate; evidence for which is being sought.

It is also clear from Fig. 8 that the reaction is influenced by the presence of Mg^{++} and Mn^{++} . (0.1 ml. Mn-Mg soln. added contained 55 μ l. Mg and 11 μ l. Mn.) Special precautions would be necessary to obtain an enzyme prep. free from traces of these metals.

The fermentation of pentose phosphoric acid was in all these respects like that of hexosemonophosphate, which has been so much studied.

Alcohol estimations

Table II. *Alcohol estimations with dried enzyme*

Substrate	Coenzyme	Activator	Time of exp. (min.)	Alcohol μ l.	CO ₂ evolved μ l.
Ribosephosph.	None	None	150	11	0
			240	0	0
"	+	None	240	69	32
"	+	Acetald.	240	475	397
"	None	Hexosediphosph.	240	26	11
"	+	"	240	369	439
Water	+	"	240	38.5	66.5
Hexosemonophosph.	None	"	310	5.5	1.5
"	+	"	310	624	682

The results show that the CO₂ output is on the whole in reasonably good agreement with the alcohol formation, on the basis of the assumption that 1 ml. pentose ester gives 1 mol. CO₂ and 1 mol. alcohol. A great many experiments and long practice with the micro-estimation would be necessary to prove the exact degree of agreement.

In view of the fact that in the one exp. with acetaldehyde 1100 μ l. of this substance were added, the degree of its removal may be regarded as fairly satisfactory, and makes it very improbable that volatile aldehydes formed in the fermentation could interfere seriously with the alcohol estimation.

The exp. of Table III shows that nearly all the ester P added is split off during the incubation, whether fermentation occurs or not (with or without cozymase).

Table III. *Phosphate balance sheet*

Vessel no. ...	1	2	3	4	5
Contents of vessel:					
A. Enzyme + KH_2PO_4 + Mg + Mn	+	+	+	+	+
B. Hexosediphosph. 0.1 ml. <i>M</i> /50	+	+	+	+	+
C. Cozymase, 0.2 mg.	—	+	+	—	+
Substrate:					
D. Ribosemonophosph. 0.3 ml.	+	+	—	—	—
E. Hexosemonophosph. 0.15 ml.	—	—	—	+	+
310 min. incubation. N_2 . 37.5°					
Inorganic P mg. added in component:					
A	1.37	1.37	1.37	1.37	1.37
B	0.00	0.00	0.00	0.00	0.00
C	0.00	0.00	0.00	0.00	0.00
D	0.06	0.06	0.00	0.00	0.00
E	0.00	0.00	0.00	0.03	0.03
Total at start	1.43	1.43	1.37	1.40	1.40
Found at end	2.16	2.21	1.53	1.81	1.87
Increase inorg. P	0.73	0.78	0.16	0.41	0.47
Organic P (acid hydrolysable, not precip. trichloroacetic acid) mg. added in component:					
A	0.00	0.00	0.00	0.00	0.00
B	0.13	0.13	0.13	0.13	0.13
C	0.00	0.01	0.01	0.00	0.01
D	0.71	0.71	0.00	0.00	0.00
E	0.00	0.00	0.00	0.38	0.38
Total at start	0.84	0.85	0.14	0.51	0.52
$\frac{\text{Increase inorganic P} \times 100}{\text{Total organic P added}}$ %	87	92	110	80	90

Loss of reducing substance on fermentation

Table IV shows the balance sheet of an experiment in which the increase of inorganic P is correlated with the appearance of a non-precipitable¹ reducing

Table IV. *Incubation of ribosephosphate with and without coenzyme*

Vessel no. ...	No coenzyme		+ Coenzyme	
	1	2	3	4
Incubation time, min.	0	330	0	330
Inorg. P, μl . (31 mg. = 22,400 μl .)	1530	2370	1530	2400
Ribosephosph. ester-P added, μl .	1020	1020	1020	1020
Increase inorg. P, μl .	no. 1	840	no. 3	870
CO_2 evolved, μl .	—	11.5	—	845
Total Cu-reducing subs. per vessel, calc. as ribose (or ribosephosph.), μl .	1031	1386	1031	342
Change during exp., μl .	+ 337		— 689	
Non-precipitable Cu-reducing subs., μl .	144	922	144	160
Change during exp., μl .	+ 778		+ 16	
Orcin- FeCl_3 test on Pb acetate supernatant liquid	Trace	+++	Trace	Negative
Periodate titration on Pb acetate supernatant liquid: I_2 equiv. of HIO_4 reduced; ml. <i>N</i> /100 I_2	8.8	30.2	8.4	18.7
Increase during exp.	21.4		10.3	
Calc. I_2 equiv. of reducing substance liberated (assumed to be ribose)*	22.6		0.5	

* Taking 1 ml. *M*/100 ribose (= 224 μl .) = 6.5 ml. *N*/100 I_2 .

¹ Using the term to mean not precipitated by neutral Pb acetate.

substance, presumably ribose, in the absence of coenzyme, and with the virtually complete disappearance of reducing substance in excess of that of the blank, when ribosephosphate is incubated in the presence of cozymase.

The results of Table IV show that pentose, as indicated by the orcin- FeCl_3 test, completely disappears from the vessel containing ribosephosphate and coenzyme after fermentation is complete (ribosephosph. gives the test as strongly as free ribose). There is an increase in the HIO_4 titration on the Pb non-precipitable fraction which does not correspond with the small change of reducing value of the same fraction. It suggests the formation of a new product during fermentation, and from the fact that this substance reduces HIO_4 it probably contains two adjacent OH groups.

Nature of the HIO_4 -reducing substance. It has been shown above that the fermentation of 1 mol. *d*-ribose-5-phosphoric acid results in the formation of 1 mol. CO_2 , 1 mol. alcohol and 1 mol. H_3PO_4 . The residue of the 5-C chain remains to be accounted for:

The most obvious choice would be glycollaldehyde, which arises during the action of Na_2CO_3 on pentoses [Fischler & Boettner, 1928] together with methylglyoxal, the latter possibly from intermediate formation of triose. Glycollaldehyde has also been suggested as an intermediate in the fermentation of pentose by the pentose-fermenting bacteria by Peterson & coworkers and by van der Lek [lit. quoted Kluyver, 1935]. Titration of the NaHSO_3 -combining substances and distillation of volatile acids revealed no significant increase, however; hence neither glycollaldehyde nor acetic acid appears as a main product. It should be possible by fixation experiments to obtain evidence of the intermediate formation of glycollaldehyde, if this occurs. This is now being tested.

Neuberg & Schwenk [1916] found that glycollaldehyde was reduced by vigorously fermenting yeast to form glycol. The following exp. however suggests that glycollaldehyde, if formed, would probably undergo dismutation under our conditions.

200 mg. dialysed dried Lebedew prep. were dissolved in 2 ml. water + 1 ml. 1.3% NaHCO_3 . 0.4 mg. cozymase was added and the soln. (1 ml.) placed in the main part of three Warburg vessels. $\text{N}_2 + \text{CO}_2$, 37.5°. The side bulbs contained 5 mg. glycollaldehyde, 5 mg. acetaldehyde and water respectively. A steady positive pressure developed only in the vessel with glycollaldehyde. It amounted to the equiv. of 100 μl . CO_2 per hr.: water control 12 μl . CO_2 per hr.

The nature of the products formed is being investigated, but it is probable that dismutation to glycollic acid and glycol had occurred, with displacement of CO_2 from the bicarbonate.

This result shows that the formation of glycol ($\frac{1}{2}$ mol.) and glycollic acid ($\frac{1}{2}$ mol.) from glycollaldehyde (1 mol.) could occur with our enzyme prep. As yet however tests for glycollic acid [Denigès, 1909] have not been successful. On the other hand, there is an increase in the HIO_4 -titratable material after fermentation, which might indicate the presence of glycol. The substance is non-Cu-reducing and is not precipitable by neutral Pb acetate and the facts are consistent with its being glycol, except that its reducing value towards HIO_4 exceeds that calc. for $\frac{1}{2}$ mol. glycol. per mol. pentose. The nature of this substance will be further investigated.

Discussion of these results will be postponed until the fate of the 2-C moiety is cleared up. In the meantime, however, attention may be drawn to the requirement for inorganic P if fermentation is to proceed, suggesting the need for formation of a diphosphate, analogous to that found in hexose fermentation. Recently Dische [1938] has made the interesting observation that *d*-ribose combined in adenosine is phosphorylated by inorganic phosphate in blood haemolysate, and that the phosphorylated ribose is afterwards broken down to a number of products, including probably triosephosphate and glycollaldehyde or glycollaldehyde phosphate. Phosphorylation and fermentative cleavage of ribosephosphates may thus occur in animal cells also.

SUMMARY

d-Ribose-5-phosphoric acid is readily fermented by Lebedew macerate of dried beer yeast with the formation of 1 mol. alcohol, 1 mol. CO₂, 1 mol. free phosphoric acid and an as yet unidentified product.

Other pentose-5-phosphoric acids are very little fermented (*d*-arabinose and xylose esters were used). The rate of fermentation of ribosephosph. is similar to that of glucose or hexosemonophosphate (Robison ester), when allowance is made for the fact that these substances both give 2 mol. CO₂ and alcohol, instead of only the one given by ribosephosph. As would be expected, intact yeast cells hardly ferment ribose or its phosphoric ester.

An enzyme preparation free from coenzyme and phosphate required the addition of cozymase and phosphoric acid before fermentation set in. An activator (hexosediphosphate, acetaldehyde, glycollaldehyde) is needed to remove the delay in onset of fermentation, as with hexosephosphate fermentation in similar preparations.

The products of the reaction with the dried enzyme, cozymase, inorg. P, Mg and Mn salts, are the same as with Lebedew extract as far as the alcohol, CO₂ and inorg. P are concerned.

The experiments are being continued to determine the nature of the remaining product. It does not reduce alkaline Cu reagent, is not precipitated by neutral Pb acetate and appears to contain two or more adjacent unsubstituted hydroxyl groups.

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