Some Properties of Fructose-1,2-cyclic Phosphates and Fructose-2-phosphates

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Fructose-1,2-cyclic phosphates(F-1,2-Ps) and fructose-2-phosphates(F-2-Ps) were synthesized according to the method of Pontis and Fischer and their properties toward acid hydrolysis were examined in detail. Fructopyranose-2-phosphate(Ep-2-P) showed exactly the same properties as those reported by Pontis and Fischer. On the other hand, a part of fructofuranose-2-phosphate(Ff-2-P) was converted into the acid-stable form during acid treatment and this substance increased progressively when the sample was stored at -20°C for a longer time. This acid-stable form resembled in its properties to the fructose phosphate obtained from UDP(2)-fructose by the action of nucleotide pyrophosphatase.

When treated with acid at room temperature, fructopyranose-1,2-cyclic phosphate(Fp-1,2-P) was almost completely hydrolyzed into fructose and inorganic phosphate, whereas only about 50% of fructofuranose-1,2-cyclic phosphate(Ff-1,2-P) was hydrolyzed into fructose and inorganic phosphate and the remainder was hydrolyzed into fructose-1-phosphate(F-1-P). On the other hand, when treated with acid at 100°C , 90% of both cyclic phosphates were hydrolyzed into F-1-P, and fructose and inorganic phosphate were formed only slightly.

In the previous papers,^{1,2)} we have reported that the properties of natural UDP-fructose isolated from Jerusalem artichoke tubers were not identical to those of UDP(1)-fructose and UDP(2)-fructose synthesized from F-1-P and F-2-P, respectively, and deduced from these results that the structure of natural UDP-fructose might be UDP(2)- α -fructofuranose whereas the structure of synthetic UDP(2)-fructose was UDP(2)- β -fructofuranose.

In the course of these investigations, we observed some anomalous properties of synthetic UDP(2)-fructose and fructose phosphate obtained from it by the action of nucleotide pyrophosphatase.

In the present paper, we report some properties of F-2-Ps and F-1, 2-Ps as synthesized by the method of Pontis and Fischer.³⁾ Ff-2-P was converted into an acid-stable form when stored at -20°C and F-1, 2-Ps were hydrolyzed mainly to give F-1-P when heated

in acid, whereas they were hydrolyzed mainly to give fructose and inorganic phosphate when treated in acid at room temperature.

MATERIALS AND METHODS

Materials. F-1-P was synthesized from fructose by the method of Pogell.⁴⁾ F-1,2-Ps and F-2-Ps were synthesized by the method of Pontis and Fischer³⁾ and purified by chromatography on a column of Dowex 1 $\times$ 4 (borate form) according to the method of Lefebvre *et al.*⁵⁾ All other chemicals were obtained from commercial sources.

Analytical methods. Total phosphate was determined by the modified method⁶⁾ of Bartlett⁷⁾ or of Hurst⁸⁾ after digestion with sulfuric acid. Acid-labile phosphate was determined by the modified method of Hurst after hydrolysis of the sample in 1 N sulfuric acid at 100°C for 15 min. Usually, inorganic phosphate was determined by the method of Hurst, but in the presence of F-1,2-P and/or F-2-P, which were hydrolyzed easily into fructose and inorganic phosphate during the time necessary for color development in the Hurst's pro-

cedure, the method of Marsh⁹⁾ was used in place of that of Hurst. In this method, the sample was treated with 1 N sulfuric acid so briefly that acid-labile phosphate compounds such as mentioned above were not hydrolyzed during this procedure.

Reducing sugars were determined by the method of Park and Johnson¹⁰⁾ with a 1/5 scale. Fructose was determined by the cysteine-carbazole method of Dische and Borenfreund¹¹⁾ with slight modifications.¹²⁾

Paper chromatography. Tôyô filter paper No. 51A was used throughout this investigation. The paper was irrigated by the descending method at 23°C. The solvent systems used were:

- A) isopropyl alcohol: ammonia: water (7: 1: 2)¹³⁾
- B) methyl cellosolve: methyl ethyl ketone: 3 N ammonia (7: 2: 3)¹⁴⁾
- C) ethyl alcohol: 1 M ammonium acetate (pH 7.5) (2.5: 1)¹⁵⁾

Phosphate and fructose compounds on the dried paper were detected by the molybdate spray reagent¹⁶⁾ and the naphthoresorcinol phosphate reagent,¹⁷⁾ respectively.

RESULTS AND DISCUSSION

Fructose-2-phosphates

Ff-2-P and Fp-2-P synthesized as described above were identical to those reported by Pontis and Fischer³⁾ in the following properties: 1) elution patterns from a column of Dowex 1 > 4(borate form), 2) relative paper chromatographic mobilities with solvent systems A) and B) (Table I), 3) response to the molybdate

spray reagent on the paper chromatogram (they gave yellow spots immediately when sprayed with the molybdate reagent).

Furthermore, Fp-2-P was completely hydrolyzed into inorganic phosphate during the Hurst's procedure and gave the hydrolysis rate constant ($k=0.0178 \text{ min}^{-1}$) at pH 4 and at 37°C (Fig. 1) similar to that ($k=0.0158 \text{ min}^{-1}$) calculated from a similar curve given in the original paper.³⁾

FIG. 1. Hydrolysis Curve of Fp-2-P at pH 4 and at 37°C.

Fp-2-P (0.7 μ mole) was incubated in 1.0 ml of 0.05 M acetate buffer (pH 4.0) at 37°C and, at intervals, 0.04 ml aliquots were withdrawn and subjected to the determination of reducing sugar. The rate constant was calculated from this curve by the equation: $k=1/t \log [a/(a-x)]$, in which a is the initial concentration of substrate and x is the concentration of product after t min.

However, Ff-2-P was not hydrolyzed completely during the Hurst's procedure. As shown in Table II, only about 60% of Ff-2-P was hydrolyzed into inorganic phosphate, whereas Pontis and Fischer³⁾ reported that this compound was completely hydrolyzed during the Fiske-Subbarow's procedure, in which less acid condition than that used in the Hurst's procedure is used. When this sample was hydrolyzed at pH 4 and at 37°C, the hydrolysis curve obtained was remarkably different from that reported by Pontis and Fischer.³⁾ As shown in Fig. 2a, about 60% of Ff-2-P was hydrolyzed during the first 30 min and the

TABLE I. R_{F1} VALUES OF Ff-2-P AND Fp-2-P IN PAPER CHROMATOGRAPHY

On the dried paper chromatogram, phosphates were detected by molybdate spray reagent and then fructose compounds were detected by naphthoresorcinol phosphate reagent, respectively. For other details, see "Paper chromatography."

Compounds	Solvent System		
	A)	B)	C)
F-1-P	1.78	2.18	1.33
F-6-P	1.78	2.40	—
F-1,6-diP	0.45	0.36	—
Ff-2-P	2.11	4.00	1.83
Fp-2-P	1.78	2.36	1.23

TABLE II. ANALYSIS OF Ff-2-P AND Fp-2-P.

For details, see "Analytical methods."

	Molar Ratio to Total-P					
	Total-P	Labile-P	Inorganic-P	Fructose	Reducing Sugar	
					before hydrolysis	after hydrolysis
Fp-2-P	1.00	1.02	0.98	1.02	0	—
Ff-2-P	1.00	1.00	0.56	1.06	0	0.97

FIG. 2. Hydrolysis Curve of Ff-2-P at pH 4 and at 37°C.

The method used was the same as in the legend Fig. 1. Fig. b) was drawn by taking the reducing value at 30 min of hydrolysis of Fig. a) as 100%. The rate constant was calculated from the curve of Fig. b).

remaining 40% was not hydrolyzed any more during the following 90 min. When these data were recalculated by taking the reducing power generated after 30 min of hydrolysis as 100%, we obtained Fig. 2b, which was, to our surprise, very similar to that reported by

Pontis and Fischer; *i.e.*, the hydrolysis rate constant calculated from Fig. 2b was $k = 0.069 \text{ min}^{-1}$, whereas that obtained from the data of Pontis and Fischer was $k = 0.059 \text{ min}^{-1}$. It may be argued that the sample used in these experiments contained some other acid-stable fructose phosphate(s) in addition to Ff-2-P. However, as the sample was shown, by paper chromatography, to be free from F-1-P and other known fructose phosphates, it may rather be considered that this sample contains some unknown acid-stable substance(s).

Furthermore, the synthetic Ff-2-P was gradually converted into an acid-stable substance when stored at -20°C for a longer time. Table III shows the analytical data of Ff-2-P which was stored at -20°C for 1, 3 and 29 months. Each stored sample was purified by paper chromatography with solvent system A) (these samples showed practically the same mobility as that of newly synthesized Ff-2-P) and analyzed as in the case of Table I. It is clear from Table III that Ff-2-P became more acid stable when it was stored for a longer time. The sample stored for 29 months did not give a yellow spot on the paper chromatogram when sprayed with the molybdate reagent.

From these facts one may consider that the synthetic Ff-2-P contains some acid-stable substance(s) and that the content of this fraction increases gradually during its storage even at -20°C . Although there is no additional data pertaining to the nature of this acid-

TABLE III. ANALYSIS OF Ff-2-P STORED AT -20°C FOR VARIOUS TIMES

For details, see "Analytical methods."

Time of storage (month)	Molar ratio to total-P				Reducing Sugar	
	Total-P	Labile-P	Inorganic-P	Fructose	before hydrolysis	after hydrolysis
1	1.00	1.00	0.61	1.05	0.11	0.97
3	1.00	0.88	0.61	1.02	0.21	0.77
29	1.00	0.72	0.00	0.96	0.04	0.69

stable substance, in the absence of evidence against the following view, Ff-2-P is supposed to be converted into an unknown acid-stable form or an acid-stable complex with the existing salts.

The properties of the acid-stable substance described above are quite similar to those of a fructose phosphate produced from UDP(2)-fructose by the action of nucleotide pyrophosphatase reported in the previous paper.²⁾ The identification of this acid-stable substance is open to a further investigation.

Fructose-1,2-cyclic phosphates

Ff-1,2-P and Fp-1,2-P synthesized as described above showed the same properties as those reported in the original paper³⁾ in the following items; 1) elution patterns from a column of Dowex 1 $\times$ 4(borate form), 2) relative paper chromatographic mobilities with solvent systems A) and B), 3) response to the molybdate spray reagent on the paper chro-

matogram (they gave yellow spots when sprayed with the molybdate reagent).

The analytical data of these cyclic phosphates summarized in Table IV indicate that Ff-1,2-P was hydrolyzed by 53% during the Hurst's procedure, whereas Fp-1,2-P was hydrolyzed completely by the same treatment. Apparently, this seems to suggest that Ff-1,2-P is more stable to acid than Fp-1,2-P.

As F-2-Ps have practically no reducing power, the fact that both cyclic phosphates have reducing power (51% for Ff-1,2-P and 25% for Fp-1,2-P) seems to be inconsistent with their structures in which the reducing OH group on C-2 is linked to phosphate. However, in the method of Park and Johnson the sample is treated in "carbonate-cyanide solution" (pH of which is about 11) at 100°C for 15 min so that there is possibility that a part of cyclic phosphates might be hydrolyzed to produce the reducing power in the course of this treatment. To confirm this, samples

TABLE IV. ANALYSIS OF Ff-1,2-P AND Fp-1,2-P

For details, see "Analytical methods."

	Molar ratio to total-P				Reducing sugar	
	Total-P	Labile-P	Inorganic-P	Fructose	before hydrolysis	after hydrolysis
Ff-1,2-P	1.00	0.98	0.53	1.00	0.51	0.73
Fp-1,2-P	1.00	1.12	1.01	1.09	0.25	0.69

TABLE V. ALKALI TREATMENT OF Ff-1,2-P AND Fp-1,2-P

To 0.5 ml of samples containing 0.7~1.0 μ mole of cyclic phosphates was added 0.5 ml of "carbonate-cyanide solution" and the reaction mixtures were heated in a boiling water bath for 15 min. From each reaction mixtures, 0.05 ml aliquots were withdrawn and subjected to phosphate determination by the method of Marsh with a 1/5 scale and by the modified method of Hurst with a 1/5 scale. Samples not treated with alkali were subjected to the same determination.

		Molar ratio to total-P	
		by Marsh	by Hurst
Ff-1,2-P	no treatment	0.06	0.59
	treated with alkali	0.55	0.67
Fp-1,2-P	no treatment	0.06	0.97
	treated with alkali	0.46	0.73

were heated in "carbonate-cyanide solution" at 100°C for 15 min and then subjected to phosphate determination by the methods of Hurst and of Marsh. Although the results somewhat varied in repeated experiments, the figures shown in Table V indicate that 55% of Ff-1,2-P and 46% of Fp-1,2-P were hydrolyzed into the so-called "true inorganic phosphate." Since in alkaline hydrolysis fructose compounds may be decomposed into various fragments, the "true inorganic phosphate" fractions may contain some extremely acid-labile phosphate compounds. However, these results clearly show that, contrary to F-2-Ps, F-1,2-Ps are at least partially decomposed during the procedure of Park and Johnson. No new fructose phosphate compound was produced as examined by paper chromatography after treatment of cyclic phosphates with "carbonate-cyanide solution."

As shown in Table IV, the reducing value of cyclic phosphates after acid hydrolysis in 0.01 N hydrochloric acid at 100°C for 15 min indicates that, contrary to our expectation, they were not hydrolyzed completely into fructose and inorganic phosphate during this

procedure. Table VI shows the molar ratios of inorganic phosphate and reducing sugar before and after hydrolysis of cyclic phosphates in 0.01 N hydrochloric acid at 100°C for 5 min (there was no difference in the results

TABLE VI. ACID HYDROLYSIS OF Ff-1,2-P AND Fp-1,2-P

To 0.9 ml of samples containing 1.3~1.5 μ moles of cyclic phosphates was added 0.1 ml of 0.1 N hydrochloric acid and the reaction mixtures were heated in a boiling water bath for 0 or 5 min, respectively. From this reaction mixtures, 0.1 ml aliquots were withdrawn and neutralized immediately with 0.1 N sodium hydroxide and then subjected to the determination of reducing sugar by the method of Park and Johnson with a 1/5 scale. Another 0.2 ml aliquots were withdrawn from the same reaction mixtures and subjected directly to phosphate determination by the modified method of Hurst.

		Hydrolysis in 0.01 N HCl	Molar ratio to total-P	
			Inorganic-P	Reducing sugar
Ff-1,2-P	0°C for 0 min		0.55	0.51
	100°C for 5 min		0.09	0.76
Fp-1,2-P	0°C for 0 min		0.94	0.29
	100°C for 5 min		0.11	0.69

whether the samples were hydrolyzed for 5 min or 15 min). This table shows that both cyclic phosphates were hydrolyzed only by about 10% into inorganic phosphate and that the remaining portion has been changed into an acid-stable compound. Taking into account of the fact that F-1-P was about 60% of reducing power as compared to free fructose,¹⁾ this acid-stable compound is supposed to be F-1-P from the values of reducing sugar after acid hydrolysis.

Table VII shows the paper chromatographic identification of hydrolysis products of cyclic phosphates when they were treated in 0.01 N hydrochloric acid at 100°C for 5 min and in 1 N sulfuric acid at room temperature for 30 min (the condition used in the Hurst's procedure). The facts that (i) inorganic

TABLE VII. PAPER CHROMATOGRAPHIC IDENTIFICATION OF ACID HYDROLYSIS PRODUCTS OF Ff-1,2-P AND Fp-1,2-P

The papers were developed with solvent system A). For other details, see the text.

	Compounds	Hydrolysis	Detection ^{R_{P1}} by phosphate	Detection by fructose	Identification
Experiment I	Ff-1,2-P	0.01 N HCl at 100°C for 5 min	0.89	—	Inorganic-P
			1.44	1.44	F-1-P
		1 N H ₂ SO ₄ at 16 °C for 30 min	0.78	—	Inorganic-P
	1.33		1.33	F-1-P	
	—		7.10	Fructose	
	F-1-P		1.67	1.67	
	Ff-2-P		2.44	2.44	
	Ff-1,2-P		6.00	6.00	
	Fructose		—	7.22	
	Experiment II	Fp-1,2-P	0.01 N HCl at 100°C for 5 min	1.00	—
1.75				1.75	F-1-P
1 N H ₂ SO ₄ at 16 °C for 30 min			—	7.25	Fructose
			1.00	—	Inorganic-P
			1.87		(F-1-P)
—			7.25	Fructose	
F-1-P			1.87	1.87	
Fp-2-P			1.87	1.87	
Fp-1,2-P			5.25	5.25	
Fructose			—	7.25	

TABLE VIII. BEHAVIOR OF Ff-1,2-P AND Fp-1,2-P AGAINST ACID TREATMENTS

Cyclic phosphates (0.12~0.13 μ mole) in a final volume of 1.0 ml were preincubated as indicated in the table, then they were subjected immediately to phosphate determination by the modified method of Hurst. Samples dissolved in distilled water showed the pH of 6~7 and were subjected to the same run.

Preincubation			Molar ratio to total-P	
Acid strength	Temperature (°C)	Time (min)	Ff-1,2-P	Fp-1,2-P
pH 6~7	24	15	0.50	0.98
	100	15	0.24	0.89
0.01 N H ₂ SO ₄	24	15	0.38	0.97
	100	15	0.07	0.10
0.01 N HCl	24	15	0.38	0.89
	100	15	0.09	0.11
without preincubation			0.49	0.93

phosphate was detected, while no fructose was detected after treatment of Ff-1,2-P in 0.01 N hydrochloric acid at 100°C for 5 min and that (ii) fructose could not be detected while inorganic phosphate was located at the position of F-1-P after treatment of Fp-1,2-P in 1 N sulfuric acid at 16°C for 30 min, were considered to be due to the relatively low sensitivity of fructose to the spray reagent used. Accordingly, Table VII shows that both cyclic phosphates were hydrolyzed into F-1-P, fructose and inorganic phosphate by either acid treatment.

In view of the results described above, it is considered that in the hydrolysis conditions used in Table VII, the cyclic phosphates were first hydrolyzed into F-1-P and F-2-Ps in various ratios and the latter were in turn hydrolyzed into fructose and inorganic phosphate as soon as they were formed. The ratios of F-1-P and F-2-Ps produced at first were calculated from the data of Tables IV and VI and the hydrolytic pathways of cyclic phosphates in acid were deduced as shown in Fig. 3. It is clear from this figure that with treatment in 1 N sulfuric acid at room tem-

perature for 15 min, 53~55% of Ff-1,2-P was hydrolyzed into Ff-2-P which was then rapidly hydrolyzed into fructose and inorganic phosphate and the remainder was hydrolyzed into F-1-P. On the other hand, Fp-1,2-P was hydrolyzed almost exclusively into Fp-2-P which was also rapidly hydrolyzed into fructose and inorganic phosphate. Now, when cyclic phosphates were hydrolyzed in 0.01 N hydrochloric acid at 100°C for 5 min, about 90% of each cyclic phosphate was hydrolyzed into F-1-P and the remaining 10% was hydrolyzed into Ff-1-P or Fp-2-P which were further hydrolyzed into fructose and inorganic phosphate.

To decide whether the difference in the hydrolytic pathways of cyclic phosphates in the above two acid treatments is due to the concentration of acid used or to the property of anions used or to the temperature during acid treatments, the cyclic phosphates were treated under various conditions. Inorganic phosphate produced was determined by the modified method of Hurst and compared with total phosphate determined by the modified method of Bartlett. The results obtained are shown

FIG. 3. Hydrolytic Pathways of Ff-1,2-P and Fp-1,2-P in Acid.

Figures in parenthesis were calculated from the data of Tables IV and VI. Fructose and inorganic phosphate (P_i) are considered to be produced through the substances enclosed by the dotted line.

in Table VIII, which indicates that neither the property of anions nor the concentration of acids cause the difference mentioned above. It is clear that the temperature during the acid hydrolysis is responsible for the difference mentioned above. Especially in the case of Ff-1,2-P, the temperature caused the difference even at neutral pH.

As in the case of glucose-1,2-cyclic phosphate,¹⁵⁾ it is generally accepted that when cyclic sugar phosphate is hydrolyzed in acid, between the two sugar phosphates to be produced, the one which is more acid stable than the other is produced more in amount. However, in the acid hydrolysis of F-1,2-Ps at room temperature, the amount of F-2-Ps formed was more than that of F-1-P formed, whereas in acid hydrolysis at 100°C, the amount of F-1-P formed was much more than that of F-2-Ps formed in agreement with the case of glucose-1,2-cyclic phosphate.

The precise mechanism of the hydrolytic pathways of F-1,2-Ps in acid is not known as yet, and offers an inviting theme for further investigations.

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