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Synthesis of D-Fructopyranose 2-Phosphate and D-Fructofuranose 2-Phosphate

BY H. G. PONTIS AND CATALINA L. FISCHER

Instituto de Investigaciones Bioquímicas 'Fundación Campomar' and Facultad de Ciencias Exactas y Naturales, Obligado 2490, Buenos Aires 28, Argentina

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Little is known of the mechanism of synthesis and degradation of inulin and other fructans. Extracts of Jerusalem artichoke, which are rich in inulin, catalyse not only the hydrolysis of inulin but also the transfer of fructose residues to and from inulin and associated oligosaccharides (Edelman & Bacon, 1951*a, b*). Edelman & Recalain (1961) have also shown that tuber extracts catalyse reversible transfructosylation from 1^F-fructosylsucrose to sucrose and presumably to higher homologues. However, one wonders if the mechanism of synthesis and degradation of inulin is not similar to those discovered for the other reserve polysaccharides, glycogen and starch. If this were the case, the degradation would be phosphorolytic, whereas a fructose nucleotide would be involved in its synthesis.

So far, two fructose-containing nucleotides have been reported: guanosine diphosphate fructose in the mould *Eremothecium ashbyii* (Pontis, James & Baddiley, 1960) and uridine diphosphate fructose in dahlia tubers (González & Pontis, 1963). In each case it was not possible to identify the position of the linkage between the fructose and the pyrophosphate group, but it was believed that position 2 of the fructose residue might be involved, i.e. both nucleoside diphosphate fructose compounds would be derivatives of fructose 2-phosphate. This sugar phosphate would also be the product of a phosphorolytic cleavage of inulin, started from the fructose end of the molecule. Thus fructose 2-phos-

phate would occupy a position in fructosan metabolism similar to that of glucose 1-phosphate in glycogen and starch metabolism.

To test these assumptions and also as a first step towards the preparation of the respective nucleoside diphosphate derivatives we considered that a synthesis of D-fructose 2-phosphate was desirable.

The synthesis of D-fructose 2-phosphate would involve the phosphorylation of the hemiacetal hydroxyl. The methods that have been described consist in allowing the aceto bromo sugar to react with silver phosphate or silver dibenzyl phosphate or silver diphenyl phosphate. However, a different and very attractive route to D-fructose 2-phosphate was suggested by the work of Khorana, Tener, Wright & Moffatt (1957) on the formation of intramolecular cyclic phosphates. Thus it could be expected that D-fructose 1-phosphate (where the hemiacetal hydroxyl group occupies a neighbouring position to the phosphate) would undergo a cyclization reaction in the presence of dicyclohexylcarbodi-imide to form a cyclic phosphate. The D-fructose 1,2-cyclic phosphate thus formed might be hydrolysed in alkali to yield D-fructose 1-phosphate and the desired D-fructose 2-phosphate. This route offers the further attraction that, as D-fructose 1-phosphate (Lehninger, Sice & Jensen, 1955) exists in solution not only in the pyranose form (I) but also in the furanose (IV), it is possible to obtain as final products the two ring forms of D-fructose 2-phosphate.

The present paper reports the synthesis of D-fructopyranose 2-phosphate and D-fructofuranose 2-phosphate by this route.

When the pyridine salt of D-fructose 1-phosphate was treated with dicyclohexylcarbodi-imide in aqueous pyridine and the reaction followed by paper chromatography with the solvent system propan-2-ol-aq. ammonia (sp.gr. 0.88)-water (7:1:2, by vol.), two compounds with the mobility of cyclic phosphates (Khorana *et al.* 1957) were formed. If the reaction was allowed to proceed further only one compound travelling near the solvent front, presumably a *N*-phosphorylurea, could be observed.

These experiments suggest that the cyclic phosphates formed have five-membered rings, since the more stable six- and seven-membered rings do not react further with dicyclohexylcarbodi-imide (Khorana *et al.* 1957). On the other hand, no phosphorylurea was obtained when the reaction was carried out in the presence of triethylamine. This is in agreement with the known fact that strong bases such as trialkylamines inhibit the reaction of cyclic diesters with dicyclohexylcarbodi-imide to form *N*-phosphorylureas (Smith, Moffatt & Khorana, 1958). Further support for the conclusion that the cyclic phosphates formed have five-membered rings was obtained by isolating them and treating them again with dicyclohexylcarbodi-imide for a longer time. In both cases, only a compound with the mobility of a *N*-phosphorylurea could be found. These experiments rule out the formation of a cyclic phosphate having a six-membered ring involving the hydroxyl groups of C-1 and C-3. Further support for this conclusion is our finding that no fructose 3-phosphate was produced during the alkaline hydrolysis of the cyclic phosphates.

Table 1. *Reaction of fructose 1-phosphate with dicyclohexylcarbodi-imide at different temperatures*

The proportions of the two cyclic phosphates were determined by measurements of the phosphate content of spots on paper chromatograms developed in solvent A. Details are given in the text.

Temperature	Reaction time (hr.)	Amount of products (% of total cyclic phosphate)*	
		R_F 0.60	R_F 0.69
30°	20	67	33
65	2	59	41
85	0.5	40	60

* As the conversion into cyclic phosphate is total, the values also indicate the yields at each temperature.

The formation of two cyclic phosphates with five-membered rings is explicable if both the pyranose and furanose forms of D-fructose 1-phosphate react with dicyclohexylcarbodi-imide. Isbell & Pigman (1937) have shown that, in the pyranose-furanose equilibrium, the proportion of the furanose form increases with temperature. Thus it would be expected that, by carrying out the reaction of D-fructose 1-phosphate with dicyclohexylcarbodi-imide at higher temperatures, the proportion of the cyclic phosphates would change. The results shown in Table 1 are in agreement with this assumption. The higher the temperature, the greater the proportion of the substance corresponding to the fast-moving compound. This evidence indicates that the pyranose form (II) is the slow-moving cyclic phosphate and that the furanose (V) is the fast one. These results are similar to those of Wright & Khorana (1958), who have shown that a greater proportion of arabinofuranose 1-phosphate than arabinopyranose 1-phosphate was formed

when the initial acetylation reaction of arabinose was carried out at higher temperature.

The oxidation of the cyclic phosphates (Fig. 1) with periodate provided further and conclusive support for their structures. The fast-moving cyclic phosphate consumed 1 mole of oxidant/mole as required for (V), and the slow-moving one consumed 2 moles/mole in agreement with (II). It was necessary to perform the periodate oxidations at neutral pH on account of the acid-lability of the cyclic phosphates. Attempts to carry out the oxidation in acetate buffer at pH 5 led to a periodate consumption similar to that of D-fructose 1-phosphate.

The separation and isolation of the cyclic phosphates on a preparative scale presented a problem. Paper chromatography with the system propan-2-ol-aq. ammonia (sp.gr. 0.88)-water (7:1:2, by vol.), and electrophoresis on borate, provided good separation, but the use of these techniques on a larger scale is rather cumbersome. Ion-exchange chromatography on Dowex 1 (borate form) gave good resolutions (Fig. 2) and could be used for separation on a preparative scale. Further, the use of ammonium borate as eluting agent, instead of the commonly used potassium borate or sodium borate (Khym & Cohn, 1953; Diedrich & Anderson, 1961), greatly simplified the recovery of cyclic phosphates (see the Experimental section for details).

The final reaction, the ring-opening of the cyclic phosphates, was conveniently followed by paper electrophoresis. It is known (Khorana *et al.* 1957) that cyclic phosphates with five-membered rings are highly labile to acid and alkali. Indeed, treatment of (II) with N-sodium hydroxide for 10 min. at 37° was sufficient for complete hydrolysis, with formation of D-fructopyranose 2-phosphate (III) and D-fructose 1-phosphate. D-Fructofuranose 1,2-cyclic phosphate (V) was even more labile. Dissolution in 0.5N-sodium hydroxide at room temperature was sufficient for opening of the ring. A minimum alkali:cyclic phosphate molar ratio of 10 was found to be necessary for opening the rings. Alkaline hydrolysis of both cyclic phosphates was always accompanied by the appearance of inorganic phosphate, which could be explained by the lability of D-fructose 1-phosphate in alkali.

No general rule for the ring-opening of unsymmetrical cyclic phosphates can be given. However, the 1,2-cyclic phosphates of glucose (Paladini & Leloir, 1952), and of galactose and xylose (R. Piras, personal communication), are hydrolysed by acid to give some 75% of the 2-phosphate and 25% of the 1-phosphate. The same proportion of secondary and hemiacetal phosphates is found in the alkali-catalysed hydrolysis of D-glucose 1,2-cyclic phosphate (Paladini & Leloir, 1952). These examples suggest that the main product of the hydrolysis of

both D-fructose 1,2-cyclic phosphates would be D-fructose 1-phosphate, on account of the greater stability of the alkyl ester linkage compared with that of the hemiacetal one. Moreover, it would be expected that the yield of the D-fructofuranose 2-phosphate should be smaller than that of the D-fructopyranose analogue on account of the known greater lability of D-fructofuranosides (Barry & Honeyman, 1952). These assumptions were supported by the results. The yield of D-fructofuranose 2-phosphate (VI) never exceeded 15%, but that of the pyranose 2-phosphate was 25–45%, both based on the respective cyclic phosphate.

Fig. 1. Periodate oxidation of cyclic phosphates: ○, D-fructopyranose 1,2-cyclic phosphate; ●, D-fructofuranose 1,2-cyclic phosphate. Details are given in the text.

Fig. 2. Ion-exchange chromatography of cyclic phosphates: D-fructofuranose 1,2-cyclic phosphate (V); D-fructopyranose 1,2-cyclic phosphate (II). The broken line represents the gradient of borate concentration. Details are given in the text.

The overall yield varied between 2 and 6 % for (VI) and between 8 and 17 % for (III), based on D-fructose 1-phosphate. D-Fructose 1-phosphate was recovered in 50–75 % yield, depending on the amount of inorganic phosphate formed. Attempts to improve the yield, especially of the fructofuranose ester, by using ammonia, pyridine, sodium carbonate or barium hydroxide for ring-opening, were unsuccessful.

Ion-exchange chromatography on Dowex 1 (borate form) again provided a good method for the separation of the hydrolysis products (Fig. 3). After alkaline hydrolysis, (V) and (II) disappeared completely (Figs. 3b and 3c respectively), and the major product formed in each case was, as expected, D-fructose 1-phosphate. The first peak is in one case D-fructofuranose 2-phosphate (VI), and in the other it is the D-fructopyranose 2-phosphate (III) overlapped by inorganic phosphate.

The D-fructose 2-phosphates have been characterized in a number of ways. The analytical data shown in Table 2 are in agreement with the formulation of the substances as (III) and (VI). D-Fructofuranose 2-phosphate could be separated well from the D-fructopyranose analogue by paper chromatography with various solvent systems (see the Experimental section: Table 4). In contrast with other fructose esters, on treatment of chromatograms with the phosphate reagent they gave yellow spots (like those from inorganic phosphate) which turn blue after heating and exposing to ultraviolet light. This is readily explained on account of the extreme acid-lability of both esters (Table 2). As shown in Fig. 4, at pH 4 and 37° about 50 % of the D-fructofuranose 2-phosphate is split in about 8 min., whereas the D-fructopyranose

Fig. 3. Ion-exchange chromatography of the products of alkaline hydrolysis: D-fructofuranose 1,2-cyclic phosphate (V); D-fructopyranose 1,2-cyclic phosphate (II); D-fructofuranose 2-phosphate (VI); D-fructopyranose 2-phosphate (III); inorganic phosphate (P_i); D-fructose 1-phosphate (F-1-P). (a) Separation with an artificial mixture; (b) and (c) alkaline hydrolysis products of (V) and (II) respectively. The extinction values corresponding to the peaks of D-fructose 1-phosphate have been multiplied by a factor of 2, to obtain their real value. The broken line represents the gradient of borate concentration. Details are given in the text.

Table 2. Analysis of D-fructose 2-phosphates

	D-Fructopyranose 2-phosphate (barium salt)*		D-Fructofuranose 2-phosphate (sodium salt)	
	(μmoles/mg.)	(μmole/μmole of fructose)	(μmoles/ml.)	(μmole/μmole of fructose)
Total phosphate	2.15	0.98	42.2	1.0
Acid-labile phosphate	2.15†	0.98	42‡	1.0
Inorganic phosphate (Fiske & Subbarow, 1925)	1.87	0.85	41	0.98
Inorganic phosphate (magnesia mixture)	0	—	0	—
Fructose	2.2	—	42	—
Reducing value	0	—	0	—
Reducing value after hydrolysis	2.15†	0.98	41.6‡	0.99
	- 83.3%		- 53.6%	

$[\alpha]_D^{20}$

* The purity was 83% calculated on the basis of the phosphate content for the dried barium salt.

† Hydrolysis at pH 4 for 120 min. at 37°.

‡ Hydrolysis at pH 4 for 30 min. at 37°.

§ Light-path 0.5 dm.; c 0.36 in water. Concentration was calculated on the basis of the phosphate content as the anhydrous barium salt.

|| Light-path 1.0 dm.; c 0.13 in water. Concentration was calculated on the basis of the phosphate content as the anhydrous disodium salt.

ester at the same pH and temperature is 50% hydrolysed in about 28 min.

The configuration of the D-fructose 2-phosphates appears to be β by comparison of their molecular rotations with those of known β -D-fructopyranosides and β -D-fructofuranosides (Table 3).

During this work, synthetic D-fructopyranose 2-phosphate and D-fructofuranose 2-phosphate were found to separate well on longer columns of Dowex 1 (borate form) (Fig. 5). This finding was made the basis of an alternative and more direct preparation of both substances. Thus D-fructose 1-phosphate was brought into reaction with dicyclohexylcarbodi-imide in aqueous pyridine as above, to give a mixture of (II) and (V). This mixture was directly hydrolysed with alkali under the conditions suitable for the ring-opening of (II). The alkaline hydrolysate was applied directly to the column and, after the run, the separated peaks were treated as above.

EXPERIMENTAL

Materials. D-Fructose 1-phosphate and D-fructose 3-phosphate were synthesized as described by Raymond & Levene (1929). Dicyclohexylcarbodi-imide was purchased from Mann Research Laboratories Inc., New York, U.S.A.

Methods. The following analytical procedures were used: that of Park & Johnson (1949) for reducing power, that of Roe (1934) for fructose, and those of Fiske & Subbarow (1925) and of Bartlett (1959) for phosphate. The last was used only for following the elution of the ion-exchange columns.

The inorganic phosphate produced after acid hydrolysis of the D-fructose 2-phosphates was measured by precipitation with ammoniacal magnesia mixture, by the procedure used by Friedkin (1950) for 2-deoxy-D-ribose 1-phosphate.

For paper chromatography, the following solvent systems were used: solvent A, propan-2-ol-aq. ammonia (sp.gr. 0.88)-water (7:1:2, by vol.) (Brown & Todd, 1953); solvent B, methylCellosolve-ethyl methyl ketone-3N-ammonia (7:2:3, by vol.) (Mortimer, 1952); solvent C, methanol-aq. ammonia (sp.gr. 0.88)-water (6:1:3, by vol.) (Bandurski & Axelrod, 1951). Whatman no. 4 paper was used (descending technique) with solvents A and B, and Whatman no. 1 paper (ascending technique) with solvent C.

Paper electrophoresis was performed with an apparatus similar to that described by Markham & Smith (1952), with Whatman no. 1 paper soaked in 0.05M-potassium borate, pH 9.2. In most runs, 1000 v (20 v/cm.) was applied for $1\frac{1}{2}$ hr.

The positions of the D-fructose phosphates and inorganic phosphate spots on paper were ascertained with the molybdate reagent as described by Burrows, Grylls & Harrison (1952) (Table 4).

All separations in ion-exchangers were performed with Dowex 1 (X 4; 200-400 mesh) resin. Dowex 1 (Cl⁻ form) resin was converted into the borate form by passing 0.8M-potassium borate until all the chloride had been displaced and was then washed with water until free from borate ions. The ammonium borate used for elutions was prepared by

neutralizing a freshly made boric acid solution with ammonia to the required pH.

Reaction of D-fructose 1-phosphate with dicyclohexylcarbodi-imide. The barium salt of D-fructose 1-phosphate was converted into the pyridinium salt by passage through

Fig. 4. Acid hydrolysis of D-fructose 2-phosphate at pH 4 and 37°: ○, D-fructopyranose 2-phosphate; ●, D-fructofuranose 2-phosphate.

Table 3. Molecular rotation of some D-fructosides

$[M]_D = [\alpha]_D \times \text{mol.wt.} \times 0.1.$	
D-Fructofuranosides	$[M]_D$
α -Methyl	+180.5*
α -Benzyl	+120*
β -Methyl	-97†‡
β' -Benzyl	-72.8*
2-Phosphate	-164.2
D-Fructopyranosides	$[M]_D$
α -Methyl	+85.5§
β -Methyl	-330
β -Benzyl	-350¶
2-Phosphate	-329

* Purves & Hudson (1937a).

† Schlubach & Bartels (1939).

‡ Bacon & Bell (1957).

§ Schlubach & Schröter (1928).

|| Hudson & Brauns (1916).

¶ Purves & Hudson (1937b).

Fig. 5. Ion-exchange chromatography of the products of alkaline hydrolysis of mixed cyclic phosphates. The symbols are as in Fig. 3. Details are given in the text.

either Dowex 50 (pyridinium form) or Dowex 50 (H^+ form) followed by neutralization with pyridine.

Pyridinium D-fructose 1-phosphate (12μ moles), dicyclohexylcarbodi-imide (120μ moles) and water (0.02 ml.) were made up to a total volume of 0.3 ml. with pyridine. The solution was kept in a stoppered tube at 30° .

In a similar experiment, 5μ l. of triethylamine (36μ moles) was added to the reaction mixture.

After suitable intervals, samples (0.01 ml.) were removed, diluted with water (0.1 ml.) and extracted with ethyl ether to remove the cyclohexylcarbodi-imide. The aqueous solutions were examined by paper chromatography in solvent A.

In the absence of triethylamine, after 1 hr. of reaction, two spots with R_F values of 0.60 and 0.69 (cyclic phosphates) could be seen, besides a spot of R_F 0.34 corresponding to D-fructose 1-phosphate. After 15 hr. the D-fructose 1-phosphate had almost disappeared and a new spot of R_F 0.88 corresponding to N-phosphorylurea had appeared. After 24 hr. only the spot of R_F 0.88 remained.

In the presence of triethylamine, after 16 hr. only the spots of cyclic phosphates could be observed.

Reaction of D-fructose 1-phosphate with dicyclohexylcarbodi-imide at higher temperatures. The procedure was slightly different from the one carried out at 30° . D-Fructose 1-phosphate (12μ moles) dissolved in 0.1 ml. of pyridine (containing 0.02 ml. of water) was heated for 5 min. at the corresponding temperature, to displace the pyranose-furanose equilibrium, before mixing with a similarly preheated solution of dicyclohexylcarbodi-imide (120μ moles) and triethylamine (36μ moles) in 0.2 ml. of pyridine. The reaction products were treated as described above. Samples of the aqueous solutions were spotted on adjacent strips of paper. After chromatography in solvent A, one strip was cut out and developed with the molybdate reagent. The cyclic phosphate spots that appeared were used as guides for cutting out the appropriate areas of the undeveloped strips. These were eluted with water, and phosphate was determined in samples. The results are recorded in Table 1.

Separation of D-fructopyranose 1,2-cyclic phosphate (II) and D-fructofuranose 1,2-cyclic phosphate (V) by ion-exchange chromatography. A mixture of cyclic phosphates (20μ moles) obtained as described above was applied to a column (1 cm. $\times$ 10 cm.) of Dowex 1 (borate form) resin. After washing the column with water, the cyclic phosphates

were eluted with a linear gradient of from 0 to $0.2M$ ammonium borate, pH 7.4 (total volume, 250 ml.). Fractions (1.6 ml.) were collected at a flow rate of 0.25 – 0.50 ml./min. Samples of every second fraction were taken and analysed for phosphate by the method of Bartlett (1959). Two well-resolved peaks were obtained (Fig. 2), the first (beginning at 145 ml. of eluting solution) corresponding to (V) and the second (beginning at 185 ml. of eluting solution) to (II). The pooled fractions containing each peak were freeze-dried and the remaining boric acid was removed by evaporation to dryness with methanol. The products so obtained were dissolved in water and adjusted immediately to pH 7 with N-ammonia or N-sodium hydroxide. Compounds (II) and (V) obtained in this manner were chromatographically and electrophoretically homogeneous. When kept frozen in solution, they were stable for at least 4 months.

Columns of the size indicated above could be used for separations of up to 700μ moles of the mixture of cyclic phosphates. The recovery, expressed as total phosphate, varied between 50 and 70% .

Reactions of D-fructopyranose 1,2-cyclic phosphate (II) and D-fructofuranose 1,2-cyclic phosphate (V). (a) Reaction with dicyclohexylcarbodi-imide. Treatment of the pyridinium salts of (II) and (V) with dicyclohexylcarbodi-imide according to the procedure described for D-fructose 1-phosphate, in the absence of triethylamine, resulted in the quantitative conversion of (II) and (V) into N-phosphorylurea in about 60 hr.

(b) Oxidation with periodate. Ammonium salts of (II) or (V) (about 2μ moles) and sodium periodate (about 9μ moles), pH 6.8 , were dissolved in 1 ml. of water. A blank was also prepared. The solutions were stored at room temperature in the dark. At intervals, duplicate samples (0.05 ml.) were removed and $1M$ -sodium hydrogen carbonate (0.05 ml.) and $4M$ -potassium iodide (0.01 ml.) were added. After 20 min. in the dark, 2% (w/v) starch (0.01 ml.) was added and the resulting mixtures were titrated against 5 mM-sodium arsenite. The results are shown in Fig. 1.

(c) Alkaline hydrolysis. Ammonium salts of cyclic phosphates (1 – 10μ moles) were evaporated to dryness *in vacuo* and the residues dissolved in the appropriate volumes of N-sodium hydroxide to give molar ratios of 1 , 2 , 5 and 10 . After 15 min. at 37° , samples were taken and examined by paper electrophoresis. Complete hydrolysis of the cyclic

Table 4. Paper chromatography and electrophoresis of D-fructose phosphates

Experimental details are given in the text. The results are expressed as the ratio of the distance travelled by the D-fructose phosphate to the distance travelled by inorganic phosphate.

	$R_{\text{phosphate}}$			$M_{\text{phosphate}}$ Electrophoresis
	Solvent A* (48 hr.)	Solvent B (17 hr.)	Solvent C (22 hr.)	
D-Fructofuranose 1,2-cyclic phosphate	5.1	—	—	0.57
D-Fructopyranose 1,2-cyclic phosphate	3.9	—	—	0.65
D-Fructofuranose 2-phosphate	2.2	3.8	1.4	0.72
D-Fructopyranose 2-phosphate	1.5	2.5	1.3	0.75
D-Fructose 1-phosphate	1.5	2.0	1.3	0.81
D-Fructose 3-phosphate	2.0	3.1	1.4	0.81
D-Fructose 6-phosphate	1.5	3.0	1.3	—
D-Fructose 1,6-diphosphate	0.2	0.2	1.0	—

* The cyclic phosphates were already well separated after 8 – 10 hr., but a longer run is convenient for obtaining better resolutions between the other D-fructose phosphates.

phosphates could be seen only in the solution having an alkali:cyclic phosphate molar ratio of 10. Other experiments with this ratio showed that the hydrolysis of (V) was completed in 3 min. and that of (II) in 10 min.

Preparation of D-fructofuranose 2-phosphate by alkaline hydrolysis of D-fructofuranose 1,2-cyclic phosphate (V). A solution of 500 μ moles of (V) was evaporated to dryness under reduced pressure (bath temperature, 30°). The residue was dissolved in 5 ml. of N-sodium hydroxide, kept at 37° for 5 min., cooled to 0° to stop the breakdown of D-fructose 1-phosphate, and applied to a column (1 cm. $\times$ 10 cm.) of Dowex 1 (borate form) resin. After a washing with water, the phosphate esters were eluted with a linear gradient of from 0 to 0.3 M-ammonium borate, pH 7.4 (total volume, 250 ml.). Fractions (1.5 ml.) were collected at a flow rate of 0.4 ml./min. Samples of every second fraction were taken and analysed for phosphate by the method of Bartlett (1959). Under the conditions of this test, 50% of (I), 82% of (V), 87% of (II), 100% of (III) and 100% of (VI) appeared as inorganic phosphate. Three peaks were obtained (Fig. 3b), the first corresponding to the desired compound. The fraction containing this peak was freeze-dried and the boric acid was removed by evaporation to dryness under reduced pressure with methanol (bath temperature, 30°). The final residue was dissolved in water and adjusted immediately to pH 7, with N-sodium hydroxide, giving 54 μ moles of D-fructofuranose 2-phosphate (10.8% yield, based on the cyclic phosphate). On paper chromatography in three different solvents, and on electrophoresis (Table 4), it gave a single spot. The corresponding analytical data are shown in Table 2.

Attempts to obtain the barium salt by the addition of barium acetate led to a nearly complete decomposition of (VI) into fructose and inorganic phosphate. It is known that Ba²⁺ and Ca²⁺ ions strongly catalyse the hydrolysis of some phosphate esters in both basic and acidic solutions (Westheimer, 1957).

D-Fructofuranose 2-phosphate, when kept frozen in solution at pH 7.5, is stable for at least 3 months.

The second and third peak corresponded to inorganic phosphate (125 μ moles) and D-fructose 1-phosphate (125 μ moles) respectively.

Preparation of D-fructopyranose 2-phosphate by alkaline hydrolysis of (II). A solution of 336 μ moles of (II) was evaporated to dryness under reduced pressure (bath temperature, 30°). The residue was dissolved in 3.3 ml. of N-sodium hydroxide, kept at 37° for 10 min., cooled to 0° and applied to a column (1 cm. $\times$ 10 cm.) of Dowex 1 (borate form) resin. After washing the column with water, the phosphate esters were eluted as described above. Two peaks were obtained (Fig. 3c), the first corresponding to the desired compound. The pooled fractions containing the peak were worked up in a similar manner to that used for D-fructofuranose 2-phosphate, giving an aqueous solution containing 76 μ moles (22.5% yield, based on the cyclic phosphate) of fructopyranose 2-phosphate. The solution was adjusted to pH 8.2 with N-potassium hydroxide, and 25% (w/v) barium acetate (0.4 ml.) was added. After 1 hr. at 0°, the precipitate of barium phosphate was centrifuged off and D-fructopyranose 2-phosphate was precipitated by the addition of 3 vol. of ethanol. After one night at 0°, the precipitate was collected by centrifuging and redissolved in water (1 ml.). Some insoluble material was centrifuged off and, to the clear supernatant, 1 vol. of ethanol was added.

After 4 hr. at 0°, the precipitate was collected by centrifuging. This procedure was repeated once more, and, finally, the precipitate was washed with aq. 80% (v/v) ethanol and ether. After drying the product *in vacuo*, the yield of barium D-fructopyranose 2-phosphate was 22.6 mg. (49.6 μ moles; 14.7% yield, based on the cyclic phosphate). It travelled as a single substance on paper chromatography in three different solvents and on electrophoresis (Table 4). The analytical data are recorded in Table 2.

Direct preparation of D-fructopyranose 2-phosphate (III) and D-fructofuranose 2-phosphate (VI). The cyclization reaction of D-fructose 1-phosphate (2.5 m-moles) was carried out as described above at 85° for 25 min. Water (130 ml.) was then added and the crystalline urea was filtered off. The aqueous filtrate was extracted three times with ether and the other layer was back-washed with water. The combined aqueous solution was then evaporated to dryness under reduced pressure (bath temperature, 30°). The residue was dissolved in 23 ml. of N-sodium hydroxide, kept at 37° for 8 min., cooled to 0° to stop the breakdown of D-fructose 1-phosphate, and applied to a column (3 cm. $\times$ 50 cm.) of Dowex 1 (borate form) resin. After washing with water, the phosphate esters were eluted with a linear gradient of from 0 to 0.4 M-ammonium borate, pH 8.8 (total volume, 4.5 l.). Fractions (15 ml.) were collected at a flow rate of 1.7 ml./min. Samples of every second fraction were taken and analysed for phosphate by the method of Bartlett (1959). Three small peaks (Fig. 5) of D-fructofuranose 2-phosphate (129 μ moles; 5.2% yield), inorganic phosphate (95 μ moles) and D-fructopyranose 2-phosphate (178 μ moles; 7.2% yield) appeared first, followed by a large peak of D-fructose 1-phosphate (1250 μ moles).

SUMMARY

1. Treatment of D-fructose 1-phosphate with dicyclohexylcarbodi-imide in aqueous pyridine in the presence of triethylamine led to the formation of D-fructopyranose 1,2-cyclic phosphate (II) and D-fructofuranose 1,2-cyclic phosphate (V).

2. Alkaline hydrolysis of (II) and (V) gave D-fructopyranose 2-phosphate and D-fructofuranose 2-phosphate respectively, besides D-fructose 1-phosphate.

3. The optical rotations of the synthetic D-fructose 2-phosphates suggest that they are the β -anomers.

4. The different D-fructose esters were shown to separate well by ion-exchange chromatography on Dowex 1 (borate form).

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Isolation of a Homologue of Glutathione and Other Acidic Peptides from Seedlings of *Phaseolus aureus*

By P. R. CARNEGIE

Department of Biochemistry, Faculty of Medicine, University of Singapore, Singapore 3, Malaysia

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In the germinating seed there is a rapid breakdown of the reserve proteins and a translocation of soluble nitrogen to the sites of synthesis of new enzymic and structural proteins of the seedling. Although there is considerable information on the production of amides and the changes in free amino acids, there is little about the structure of the peptide-like compounds that are known to be present (Danielsson, 1951; Folkes, 1959).

The seedlings of *Phaseolus aureus* chosen for this study are an important vegetable in the Asian diet. A preliminary fractionation of the expressed juice from these seedlings on diethylaminoethyl-Sephadex A25 showed that a complex mixture of acidic peptide-like compounds was present and that one of the peptides contained a disulphide group (Carnegie, 1963*a*). Its isolation is described in this

paper and evidence in the following paper shows that it has the structure bis- γ -L-glutamyl-L-cystinyl-bis- β -alanine, called homogluthathione (Carnegie, 1963*b*). Some of the properties of other peptide-like compounds are also discussed.

One of the main problems in the isolation of oligopeptides from seedlings is their separation from much larger amounts of free amino acids. Porath (1960) showed that it was possible to separate glutathione from isoleucine by gel-filtration chromatography on a column of dextran gel with *N*-acetic acid as solvent. By using his procedure it was possible on a column of Sephadex G25 to separate homogluthathione from the relatively large amounts of acidic amino acids and also from dipeptides present in the acid eluate from the anion-exchange resin. Separation of peptides from