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Note

The preparation of sucrose monophosphates from dried mixtures of sucrose and sodium phosphate

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Phosphate esters of saccharides are important biochemicals, some of which can be isolated (often in low amounts and with difficulty) from natural sources [1] or enzymatically prepared in vitro with a suitable kinase. Chemical methods of preparation include direct esterification using phosphoric acid (which can produce artefacts, including degradative products) [2] and indirect esterification by selective exposure of hydroxyl groups followed by their reaction with a phosphorylating reagent [3,4], this usually requiring several steps.

During studies concerned with identifying factors affecting stabilities of biological standards, we have observed that in preparations containing hydroxylated organic compounds and sodium phosphate buffer salts, phosphomonoester formation is promoted at low water activities [5,6]. This methodology therefore provides simple and direct routes to sugar (and other) phosphates and using these procedures we have described [7] the preparation and characterisation of the four isomeric monophosphates of α,α'-trehalose one of which, the 6-isomer, occurs in yeast [1]. In the case of sucrose phosphates, the 6′-isomer is present in sugar-beet leaves where it is involved in sucrose biosynthesis [8] and it has been prepared enzymatically in vitro (as has the 6-isomer) [9–11]. A definitive chemical synthesis of sucrose-6′-phosphate has been described by Buchanan and co-workers [11]. In this report we describe its preparation together with its isomers from dried solutions of sucrose and sodium phosphate salts.

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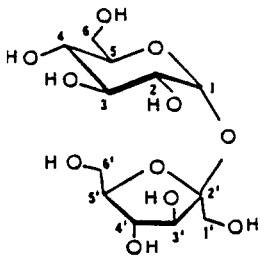

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Sucrose (**1**) when freeze-dried from sodium orthophosphate buffer around neutral pH (a range from 5.5 to 8.1 has been studied) and with a residual moisture content of 5–10% produced, especially after warming, products possessing chromatographic characteristics of phosphomonoesters (*A* to *J*, Fig. 1). Their formation was more rapid and more extensive at lower pH and also when orthophosphate was replaced by metaphosphate and these observations are similar to those previously noted [5–7]. Treatment, with alkaline phosphatase, of the products eluting (Fig. 1) between 23 and 43 min converted > 90% of the organic phosphorus to inorganic orthophosphate with the simultaneous formation of (mainly) sucrose, confirming that the majority of the products were monophosphate esters of this disaccharide. A control sample lacking enzyme was unaffected. However, enzyme digests of product mixtures at pH 5.5 contained in addition to sucrose, both glucose and fructose (ca. 15% of each were formed in preparations heated at 80°C for 5 days). Free glucose and fructose (identified chromatographically) were also present in heated, but unfractionated mixtures — after 24 h ca. 2% of each had formed. These results demonstrate the high acid lability of sucrose, even at pH 5.5. The monosaccharide phosphates may result from hydrolysis of the disaccharide and subsequent phosphorylation of the so formed monomers and from hydrolysis of the sucrose phosphates themselves.

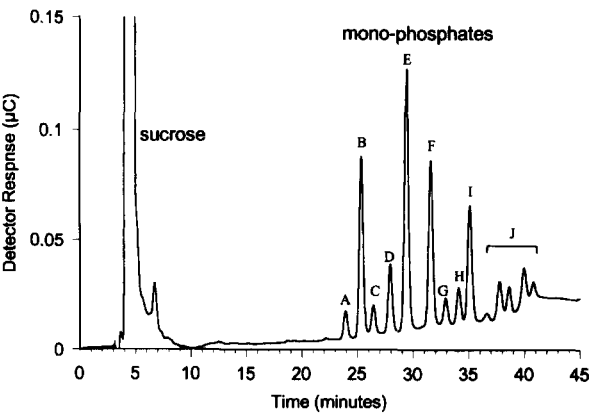

Fig. 1. Chromatogram obtained on Dionex HPLC from sucrose after drying from sodium metaphosphate solution at pH 5.5 and subsequent heating. For details see Experimental Section.

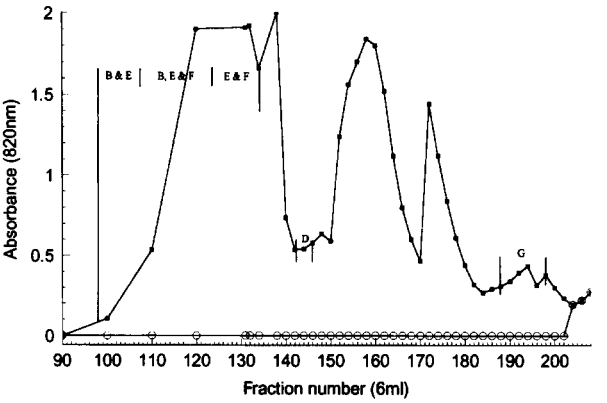

Fig. 2. Phosphorus profile after fractionation of sucrose phosphate mixture on AG1 ion-exchange column, ■–■ total phopsphoprus, ○–○ inorganic phosphorus. The fractions containing *B*, *E* and *F* and *D* and *G* are indicated. For further details see Experimental Section.

The sucrose phosphates could be conveniently prepared by heating freeze-dried solutions of sucrose and sodium metaphosphate (pH 5.5, 80°C, for 5 days). This treatment produced a mixture which, on the basis of HPLC analysis contained a total of 17% phosphomonoesters relative to sucrose in the following ratios: 3 (*A*), 14 (*B*), 3 (*C*), 8 (*D*), 22 (*E*), 14 (*F*), 3 (*G*), 5 (*H*), 14 (*I*), and 14 (*J*).

Separation of these products from neutral species and from inorganic phosphate was achieved by fractionation of the mixture on AG1 anion-exchange resin which also resulted in partial separation of the isomers as indicated in Fig. 2. This organic phosphorus-containing fraction after drying contained 6.3% by weight phosphorus (calculated for sucrose monophosphate esters P, 6.8%). Assuming that all the organic phosphorus was present as phosphate esters of sucrose, then the calculated total yield is 27%. The discrepancy between this value and that obtained from HPLC is probably due to different molar detector responses as previously noted in the case of trehalose and its phosphates [7].

Eight components of the mixture were obtained by preparative chromatography, using the Dionex HPLC, of suitable fractions from the AG1 separation. The structures of the isolated components were deduced from their ^{1}H NMR spectra. These spectra together with that of sucrose are shown in Fig. 3 with their chemical shifts and coupling constants tabulated in Table 1.

Seven of the isolated components, viz. *A*, *B*, *D*, *E*, *F*, *G*, and *H*, all produced spectra expected from derivatives of sucrose; in particular each spectrum contained a single anomeric proton (at ca. 5.4 ppm, $J \sim 4$ Hz) similar to H-1 of sucrose. The eighth component, *I*, appeared to be a mixture of a sucrose derivative and a monosaccharide since its spectrum contained two additional anomeric resonances which could be assigned to an α,β-mixture of a reducing sugar. Phosphorylation of a hydroxyl group in oligosaccharides usually results [7,12] in a downfield shift of the α-proton of up to 0.55 ppm with smaller (up to 0.2 ppm) downfield shifts experienced by other protons present in that residue with those situated β to the phosphate ester usually greatest affected.

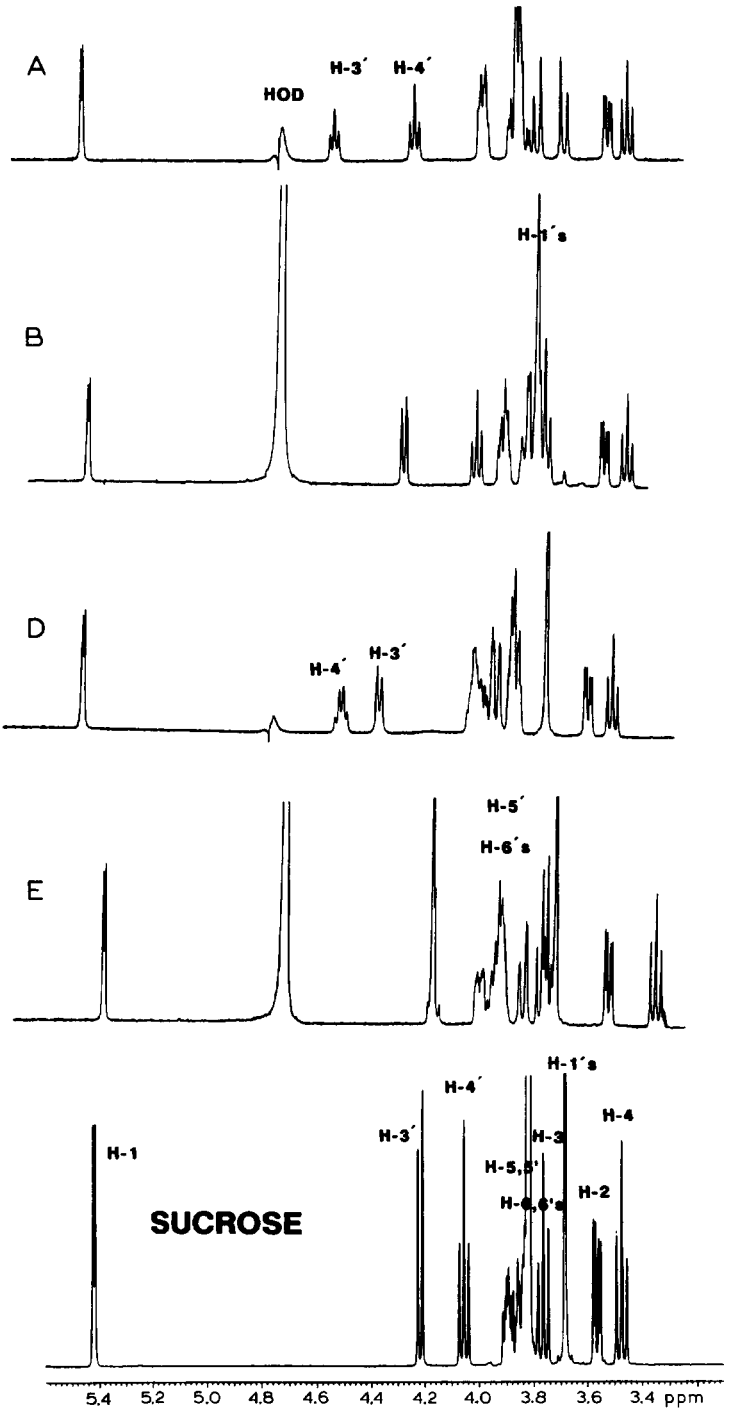

Fig. 3. ^{1}H NMR spectra of sucrose and of *A*, *B*, *D*, *E*, *F*, *G*, *H*, and *I*. The positions of resonance of those protons of major relevance are indicated.

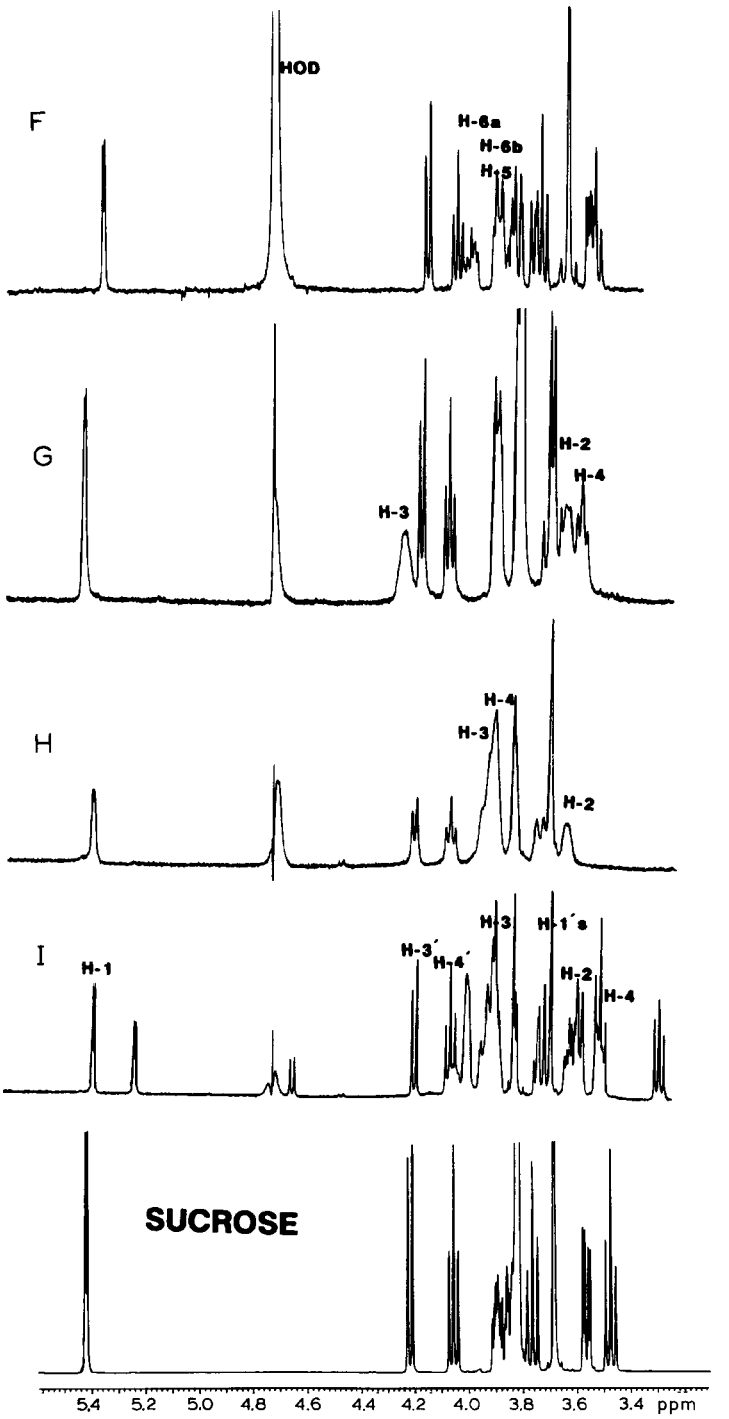

Fig. 3 (continued).

Table 1
^{1}H Chemical shifts (ppm from Me_4Si) and ^{1}H–^{1}H coupling constants (Hz) for *A*, *B*, *D*, *E*, *F*, *G*, *H*, and *I* (NH_4^+ salts) in D_2O at 500 MHz and 30°C. Included for comparison are the values for sucrose. For *I*, the observed chemical shifts (and coupling constant) are listed in footnote [a] together with tentative assignments — those resonances attributed to the sucrose derivative are in bold type

	Sucrose	*A*	*B*	*D*	*E*	*F*	*G*	*H*
H-1	5.42	5.45	5.43	5.45	5.38	5.35	5.42	5.40
H-2	3.56	3.54	3.54	3.58	3.51	3.56	3.66	3.64
H-3	3.77	3.82	3.76	3.79	3.82	3.74	4.26 [b]	3.95
H-4	3.47	3.46	3.44	3.49	3.34	3.52	3.61	3.80
H-5	3.90	3.98	3.90	3.92	3.92	3.93	3.91	3.96
H-6a	X	X	X	X	X	4.00	X	X
H-6b	X	X	X	X	X	3.89	X	X
H-1′a	3.69	3.69	3.79	3.74	3.72	3.67	3.69	3.71
H-1′b	3.69	3.79	3.79	3.74	3.72	3.67	3.75	3.71
H-3′	4.22	4.53b	4.27	4.35	4.15	4.15	4.20	4.21
H-4′	4.05	4.24	4.01	4.49 [b]	4.15	4.05	4.10	4.08
H-5′	3.86	3.98	3.90	3.97	3.92	3.86	3.91	3.93
H-6′a	X	X	X	X	3.99	X	X	X
H-6′b	X	X	X	X	3.92	X	X	X
X	3.80–86	3.81–90	3.76–84	3.82–95	3.74–78	3.75–85	3.79–86	3.73–93
$J_{1,2}$	3.5	3.6	3.4	3.9	3.9	3.9	3.0	
$J_{2,3}$	10.0	9.7	10.2	9.8	9.8	10.1	9	
$J_{3,4}$	9.1	9.3	9.3	9.9	9.8	9.8	9	
$J_{4,5}$	9.8	9.7	10.2	9.9	9.9	9.9	9	
$J_{1',1'}$		12.9					12.7	
$J_{3',4'}$	8.8	8.3	8.8	8.8		8.8	8.8	8.8
$J_{4',5'}$	8.3	8.3	8.3	~8		8.8	8.3	8.8

[a] Chemical shifts and (coupling constants) observed for *I*: **5.41 (3.9 Hz) H-1**; 5.24 (3.4); 4.67 (8.3); **4.22 (8.8 Hz) H-3′**; **4.08 (8.8 Hz) H-4′**; 4.02; 3.98–3.90; **3.87, H-3**; 3.84; 3.75; **3.71, H-1′a,1′b; 3.63 (9.3, 3.9 Hz) H-2**; 3.61; 3.55–3.53; **3.52, H-4**; 3.31 (9.3).
[b] ^{1}H–^{31}P couplings of 8–9 Hz indicated for these resonances.

Protons of the non-phosphorylated residues normally resonate at or about their chemical shifts values in the parent sugar. Analysis of the spectra obtained from the isolated products thus permits the position of phosphorylation to be determined.

The spectra of *A*, *B*, *D*, and *E* (Fig. 3) all contained resonances characteristic for an unsubstituted α-D-glucopyranosyl residue, viz. a triplet at ca. 3.47 ppm (H-4) and a quartet at ca. 3.56 ppm (H-2). H-4 and H-2 are observed at these shift values in sucrose (Table 1). *F*, *G*, *H*, and *I* had their H-4 and H-2 resonances shifted to lower fields. Consequently, *A*, *B*, *D*, and *E* are probably phosphorylated in the fructosyl residue and *F*, *G*, *H*, and *I* in the glucosyl residue.

The spectrum of *A* also contained a pair of triplets at 4.53 and 4.24 ppm, respectively assigned (confirmed from COSY or TOCSY spectra) to H-3′ and to H-4′ and shifted by +0.31 and +0.19 ppm from their values in sucrose. These data accord with sucrose-3′-phosphate; the appearance of H-3′ as a triplet resulting from its coupling to phosphorus.

For *B*, H-3′ and H-4′ (and other protons except H-1′a and H-1′b) are observed at shifts and as multiplets similar to sucrose. The 1′ protons however are deshielded by 0.1 ppm and this accords with sucrose-1′-phosphate and *B* is therefore assigned this structure.

For *D*, H-3′ and H-4′ appeared respectively as a doublet and as a quartet deshielded by 0.13 and 0.44 ppm relative to sucrose. Consequently *D* can be identified as sucrose-4′-phosphate; the appearance of H-4′ as a quartet resulting from coupling to phosphorus.

E is likely to be the 6′-isomer and in agreement with this assignment are the downfield shifts (0.1–0.2 ppm) of H-6′a, H-6′b, H-5′, and H-4′. H-4′ was in fact superimposed on H-3′ resulting in a loss of fine structure for these signals. It is noteworthy that H-4 appeared 0.13 ppm *upfield* from H-4 of sucrose presumably as a result of the 6′-phosphate group. Effects across phosphorylated disaccharide residues (e.g., in trehalose-2-phosphate) have been observed previously [7]. The spectrum obtained for *E* is identical to that of sucrose-6′-phosphate (Sigma) thereby confirming its identity.

F is identified as sucrose-6-phosphate because of the downfield shifts (0.1 to 0.2 ppm, relative to sucrose) of H-6a, H-6b, and H-5 as expected from the presence of a 6-phosphate group [7,12].

In the spectrum obtained from *G*, H-3 was poorly resolved and appeared as the lowest field non-anomeric resonance at 4.26 ppm, 0.49 ppm downfield from its value in sucrose. H-2 and H-4 were also deshielded by 0.10 and 0.14 ppm. Protons of the fructose residue appeared at chemical shifts similar to sucrose. These observations accord with *G* being sucrose-3-phosphate.

H also produced a poorly resolved spectrum possibly because of the overlap of several sets of coupled signals. However, it is apparent that H-4 and H-3 had shifted to lower field, respectively, by 0.33 and 0.18 ppm consistent with the presence of a 4-phosphate group. As for *G*, the fructosyl protons were observed as multiplets and at values expected from an unsubstituted residue. *H* is therefore identified as sucrose-4-phosphate.

As mentioned, *I* appeared to be a mixture of two components (present in about equal amounts). The anomeric resonance at 5.41 ppm can be assigned to H-1 of a sucrose derivative the other anomeric protons to an α,β mixture of a reducing sugar. By a process of elimination *I* is likely to contain sucrose-2-phosphate and this is borne out, to some extent, by the downfield shifts relative to sucrose, for H-2, H-3, and H-4 and by the observations of other resonances at values similar to sucrose. The reducing component of this mixture has not been identified; however, it would appear to possess an unsubstituted 4 hydroxyl group by virtue of the triplet observed at 3.31 ppm which may be assigned to H-4(α) of such a residue.

In all of the spectra the chemical shifts and the coupling constants of protons not specifically referred to are as expected from proposed structures. Although not measured directly, $^{31}P-^{1}H$ couplings are indicated (Fig. 3) and the magnitude estimated (Table 1) from the multiplicity of some of the resonances involved. These are in agreement with the proposed structures.

H-1′a and H-1′b were coincident in most of the spectra appearing as a singlet. In the 3- and 3′-isomers however, H-1′a and H-1′b were observed as a pair of doublets ($J > 12$ Hz) of an A-B system indicating restricted rotations around the 1′-carbon when phosphate groups are located at these positions.

To summarise, when dried from phosphate buffer, sucrose can produce a mixture of

its monophosphate esters together with, at mildly acid pH, smaller amounts of glucose, fructose and their phosphates. The components of this mixture have been isolated by ion-exchange chromatography and, for the disaccharide esters, the position of phosphorylation has been determined by ^{1}H NMR spectroscopic analysis. One component of the mixture was isolated mixed with an unidentified reducing sugar phosphate. The relative yields of the isomers indicated that, as expected on steric grounds, the three primary phosphates (*B*, *E*, and *F*) are the most abundant products.

As well as providing a direct method for the preparation for sucrose monophosphates, their formation in dried phosphate buffers has implications when sucrose is used, in these buffers as for example an excipient in pharmaceutical preparations or standards. The presence of sucrose phosphates in such preparations may lead to further reactions or effect assays for biologically-active molecules. In addition the hydrolysis, under mildly acidic conditions, of the interglycosidic linkage producing glucose, fructose and their phosphates needs careful consideration especially as *aldehydo-* and *keto*-sugars can condense with amino functional groups present in peptides and proteins giving, initially, Schiff's bases [13,14]. Such chemical modifications may have major consequences on the biological properties of active ingredients.

1. Experimental

For general methods including analytical HPLC, ^{1}H NMR spectroscopic methods and reagents used see [7].

Preparative HPLC was performed on a PA1 column (9 × 250 mm) Dionex, UK, using 100 mM NaOH and a NaOAc gradient (160–250 mM over 30 min) and a flow rate of 3 mL/min. Fractions (1 mL) were collected after the passage of the eluent through an ion-membrane suppressor (Dionex) and the fractions were then inspected using the analytical HPLC method.

Reaction of sucrose with sodium metaphosphate at pH 5.5.—Sucrose (2.0 g) in a glass container was dissolved in aqueous sodium metaphosphate (20 mL, 1 M, pH 5.5) and lyophilised under ambient conditions for 20 h. The container was then sealed and heated at 80°C for 5 days. Examination of the straw-coloured syrupy mixture by HPLC indicated 17% conversion to monophosphate esters. The mixture was dissolved in water (200 mL) and was loaded onto a column of AG1-X8 anion-exchange resin (acetate form) and the column was then eluted with a gradient (0.3–0.6 M) of NH_4OAc. Fractions (6 mL) were collected and assayed for phosphorus — total and inorganic [15]. The amount of organic phosphorus (49 mg) indicates a 27% yield (assuming the products to be sucrose phosphates). The fractions were also examined by Dionex HPLC and, as indicated in Fig. 2, the first phosphorus-containing fraction contained mixtures of *B*, *E*, and *F* with *B* and *E* partially separated from *E* and *F*. Later fractions contained mixtures of the remaining components of varying compositions with the exception of fractions 142–146 which contained *D* and fractions 188–198 which contained *G*, both > 95% purity by HPLC. The total mixture contained 6.3% w/w phosphorus (calculated for sucrose monophosphate P 6.8%)

The *B*, *E*, and *F* containing fraction (10 mg) in 0.1 M NaOH (0.1 mL) was then

chromatographed on a PA1 column (9 × 250 mm) and the components were isolated homogeneous by HPLC. Further purification (desalting) was then carried out as described below. The later-eluting fractions from the AG1 column were similarly fractionated and their individual components isolated. In this manner *A, B, D, E, F, G, H*, and *I* (see Fig. 1) were isolated in 0.5 to 2 mg quantities (as determined from their phosphorus content).

Desalting.—The individual components obtained from fractionation by Dionex HPLC contained inorganic sodium salts and these were removed in each case by loading a dilute aqueous solution of the phosphate ester onto a column (12 × 1 cm) of Macro-Prep High Q (BioRad) anion-exchange support (acetate form) and washing off the inorganic sodium salts with 5 bed volumes of water. Subsequent elution with 0.7 M aqueous NH_4OAc removed the phosphate esters and this solution was then lyophilised several times to remove most of the NH_4OAc. The products, obtained as amorphous white solids, were further purified (to remove traces of residual inorganic ammonium salts) by chromatography on a Bio-Gel P2 polyacrylamide as described previously [7].

Digestion with alkaline phosphatase.—The phosphate ester mixture isolated from the AG1-column (3 mg) was dissolved in aqueous 0.05 M $(NH_4)_2CO_3$–0.01% NaN_3, pH 8.2 (1 mL), and divided into two. One portion was treated with alkaline phosphatase (50 units), the other serving as a control. These solutions were maintained at 37°C and assayed for total and inorganic phosphorus and were also inspected by Dionex HPLC analysis.

Reaction of sucrose with sodium orthophosphate at pH 5.5.—This was carried out essentially as described for metaphosphate, except that in this case sodium orthophosphate buffer was used. Less reaction occurred, but the products formed were indistiguishable (HPLC, P content, susceptibility to alkaline phosphatase) from those isolated from the reaction with metaphosphate.

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