Recharacterization of fungal dinucleoside polyphosphate (HS3)

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Three polyphosphorylated dinucleosides given the pseudonyms of HS3, HS2, and HS1 that were erroneously described as diguanosine polyphosphates (LéJohn, H. B., Cameron, L. E., McNaughton, D. R. & Klassen, G. R. (1975) *Biochem. Biophys. Res. Commun. 66*, 460–467) have been repurified and partially recharacterized. They have proved to be extremely complex molecules; chemical (HCl and KOH hydrolysis), physical (ultraviolet-light spectral analysis and ion-exchange chromatography), and enzymic (nucleotide pyrophosphatase and bacterial alkaline phosphatase hydrolysis) studies showed that (*i*) all three HS compounds are uracil rich and (*ii*) only HS3 contains a purine nucleoside and glutamate. The partial structure of HS3 was deciphered as a moiety of ADP – sugar X – glutamate (the mode of attachment of glutamate is obscure) that is covalently linked to another moiety composed of UDP, mannitol, and four phosphates. Sugar X had chromatographic characteristics of ribitol, but the chromatographic isolate also contained a ninhydrin-sensitive entity presumed to be an amino group. Sugar X, therefore, may be an amino sugar polyol. Only the general chemical compositions of HS2 and HS1 were determined. Each contained two uridines and HS2 had 10 phosphates whereas HS1 had 12.

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Nous avons repurifié et recaractérisé partiellement trois dinucléosides polyphosphorylés, nommés HS3, HS2 et HS1, que nous avions d'abord décrits comme étant des polyphosphates de diguanosine (LéJohn, H. B., Cameron, L. E., McNaughton, D. R. et Klassen, G. R. (1975). *Biochem. Biophys. Res. Commun. 66*, 460–467). Ces composés se sont avérés des molécules extrêmement complexes. En effet, les études chimique (hydrolyse avec HCl et KOH), physique (analyse spectrale en lumière ultraviolet et chromatographie échangeuse d'ions) et enzymatique (hydrolyse avec nucléotide pyrophosphatase et phosphatase alcaline bactérienne) ont montré que (*i*) les trois composés HS sont riches en uracile et (*ii*) que seul HS3 contient un nucléoside purique et du glutamate. Nous avons déchiffré partiellement la structure de HS3: la molécule comporte une fraction ADP – glucide X – glutamate (le mode d'attachement du glutamate n'est pas clair) reliée de façon covalente à une autre fraction contenant de l'UDP, du mannitol et quatre phosphates. Le glucide X possède les caractéristiques chromatographiques du ribitol, mais le composé isolé par chromatographie contient aussi une entité sensible à la ninhydrine que nous croyons être un groupe aminé. Le glucide X serait donc un sucre aminé polyol. Seule la composition chimique générale de HS2 et HS1 est établie. Chacun contient deux uridines et HS2 a 10 phosphates et HS1, 12.

[Traduit par le journal]

Introduction

Two years ago, we assigned the pseudonyms HS3, HS2, and HS1 to three polyphosphorylated compounds isolated from a freshwater mould *Achlya* and tentatively called them diguanosine polyphosphates (1). They were shown to inhibit the enzymic activities of DNA-dependent RNA polymerases I, II, and III isolated from the same organism (2). This communication details our method of isolation, purification, and chemical recharacterization of these compounds. They are not diguanosine polyphosphates. HS3 was found to be a glutamyl–ADP–sugar moiety covalently linked in some unknown way to a UDP–sugar–tetraphosphate.

A dinucleotide similar to HS3 from *Achlya* has been isolated from cultured CHO cells and partially characterized (3, 4). Possible physiological roles for HS compounds in eukaryotes have been considered in recent communications (4, 5, 6) and further elaborated in the accompanying reports (7, 8, 9).

ABBREVIATIONS: uv, ultraviolet; TCA, trichloroacetic acid; TEAB, triethylammonium bicarbonate; CHO, Chinese hamster ovary.

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Materials and Methods

Organism

The conditions for growing *Achlya sp.* (1969) have been described (10).

Biochemicals

Common biochemicals were obtained from Sigma. [^{32}P]Orthophosphate was obtained from Amersham/Searle. Sephadex

G-10, G-30, and A-25 were obtained from both Sigma and Pharmacia Chemical Co. ppGpp and pppGpp were kindly supplied by M. Cashel.

Enzymes

Bacterial alkaline phosphatase, nucleotide pyrophosphatase, and D- and L-amino acid oxidases were purchased from Sigma.

Chromatography

Paper chromatography was performed with Whatman No. 1 and orange ribbon C (Schleicher and Schuell Co.) paper. Thin-layer chromatography was performed with PEI-cellulose and thin-layer cellulose (Cel 300/uv) plastic-backed sheets obtained from Brinkmann. The solvents used were solvent I, 1.5 M KH_2PO_4 pH 3.65 (modified solvent I consisted of 1.0 M KH_2PO_4 pH 3.65); solvent II, 3.3 M ammonium formate in 4.2% boric acid adjusted to pH 7 with concentrated NH_4OH; solvent III, 200 ml each of 0.5 M Na_2HPO_4 and NaH_2PO_4, 20 ml n-propanol, and 1200 g ammonium sulphate brought to 2 ℓ with distilled H_2O; solvent IV, 1-butanol – isobutyric acid – H_2O – ammonium hydroxide (10:5.3:3:0.3); solvent V, saturated $(NH_4)_2SO_4$ – 0.1 M sodium acetate – isopropanol (75:19:2); solvent VI, isopropanol – formic acid – H_2O (80:4:20); solvent VII, n-propanol–water (7:3); solvent VIII, ethyl acetate – acetic acid – formate – water (9:1.5:0.5:2); solvent IX, n-butanol – acetic acid – water (3:1:1); and solvent X, ethyl acetate – pyridine – H_2O (top phase) (2:1:2).

Nucleotide Analysis

HS molecules were digested with acid or enzymes (described below) and analysed for nucleobase and nucleoside components by chromatography. One-dimensional paper chromatography was performed with orange ribbon C paper in solvent III. Two-dimensional chromatography was carried out on thin-layer cellulose sheets with solvent IV in the first and solvent V in the second dimension. Localization of nucleobases and nucleosides was by uv light.

Spectral Analysis

The HS compounds were spectrally analysed at pH 1 using 0.01 N HCl pH 7 in 0.01 M sodium phosphate buffer and at pH 12 in 0.01 N KOH.

Phosphate Analysis

Total organic phosphate was estimated after ashing by the method of Lowry *et al.* (11). Phosphorylated substances were detected and identified after chromatography by autoradiography when ^{32}P-labelled HS compounds were used. Kodak RP-14 Royal X-Omat films were exposed to the chromatogram in the dark for 24–48 h before development. They were then quantitated by cutting the corresponding areas on the chromatogram and determining the radioactive content by the use of liquid scintillation. ^{32}P Radioactivity of HS or components fractionated by column chromatography was measured by the Cerenkov method (12).

Amino Acid Analysis

Approximately 1 A_{260} unit of HS3 was hydrolysed in a sealed tube with 6 N HCl at 105°C for 20 h after which excess acid was removed by evaporation *in vacuo*. The residue was washed several times with water and redissolved in a small volume of water. Analysis was performed by two-dimensional thin-layer chromatography on thin-layer cellulose plates with solvent VI in the first dimension and then solvent VII in the second dimension. Localization of amino acids was accomplished using freshly prepared ninhydrin spray (3% in ethanol) with overnight incubation to allow spots to appear. Quantitation and identification of amino acids were also carried out with a Beckman model 121 amino-acid analyser.

The optical isomeric form of the amino acid was determined using D- and L-amino acid oxidases. About 3 A_{260} units of HS3 was acid hydrolysed and approximately 1 A_{260} unit of hydrolysate was treated with the specific oxidase (1 μg/ml) in citrate buffer pH 6.5. Samples were then chromatographed one dimensionally in solvent VI and amino acids located with ninhydrin spray.

Carbohydrate Analysis

Sugars were estimated by the periodate method (13) after acid hydrolysis of fragments. Ribose was quantitated by the orcinol reaction (14). For the estimation of ribose in uridine, the procedure of Haavaldsen *et al.* was followed (15). Prior to sugar analysis, 5–10 A_{260} units of HS3 (or fragments) was hydrolysed *in vacuo* with 1 N HCl at 105°C for 20 h. Excess acid was removed by evaporation; the residue was redissolved in water and mixed with activated Dowex-50. After centrifugation for 10 min, the supernatant was removed, evaporated to dryness, and dissolved in 25 μl of distilled H_2O. Samples were chromatographed one dimensionally on Whatman No. 1 paper in either of solvents VIII, IX, or X. Localization of sugars on paper was performed with specific indicators as described by Bailey (16). The indicators were sodium periodate – permanganate (M1), p-anisidine (M2), bromocresol purple (M3), vanillin (M4), benzidene–TCA (M5), and ninhydrin (M6).

Enzyme Digestions

HS compounds or their component parts were hydrolysed by a variety of enzymes. Bacterial alkaline phosphatase and nucleotide pyrophosphatase were used at a concentration of 1 μg/ml. Usually, 1 μg enzyme was used for every single A_{260} unit of HS or fragment. Enzymes were incubated at 37°C in 0.2 M Tris–HCl pH 7.5 buffer for various times as specified in table legends and text. Nucleotide pyrophosphatase reaction mixture contained 0.01 M $MgCl_2$.

Chemical Hydrolysis

(a) Alkali

HS3 was incubated with 0.33 N KOH for 20 h followed by neutralization with KH_2PO_4 and fractionation on Sephadex A-25.

(b) Acid

HS3 and fragments were hydrolysed by either 1 N or 6 N HCl. The types of products obtained depended on the length of treatment (1–20 h) and the strength of acid used.

Column Chromatography

Columns of superfine Sephadex G-10, G-25, and A-25 were used to fractionate enzymic and chemical digests of HS3 or fragments of HS3. Details of this procedure are given in the appropriate sections in the text and figure legends. The separated components were collected by pooling fractions beneath designated peaks, concentrating to 1–2 ml by evapomix, and desalting on a Sephadex G-10 column (1 × 100 cm) equilibrated with 50 mM TEAB buffer pH 8.0. Fractions containing desalted components were pooled, evaporated to dryness, washed, and resuspended in known volumes of distilled water.

Results

Isolation and Purification of HS Compounds

Achlya was grown as 15L cultures under forced aeration as described (2) to midlog growth phase (13–14 h) at 28°C before extracting HS compounds from the mycelia. The mycelia were recovered from the growth medium by suction filtration on Whatman No. 1 filter paper, washed with distilled water, sucked dry, and then weighed. For every gram compressed wet weight of mycelia, 1 ml

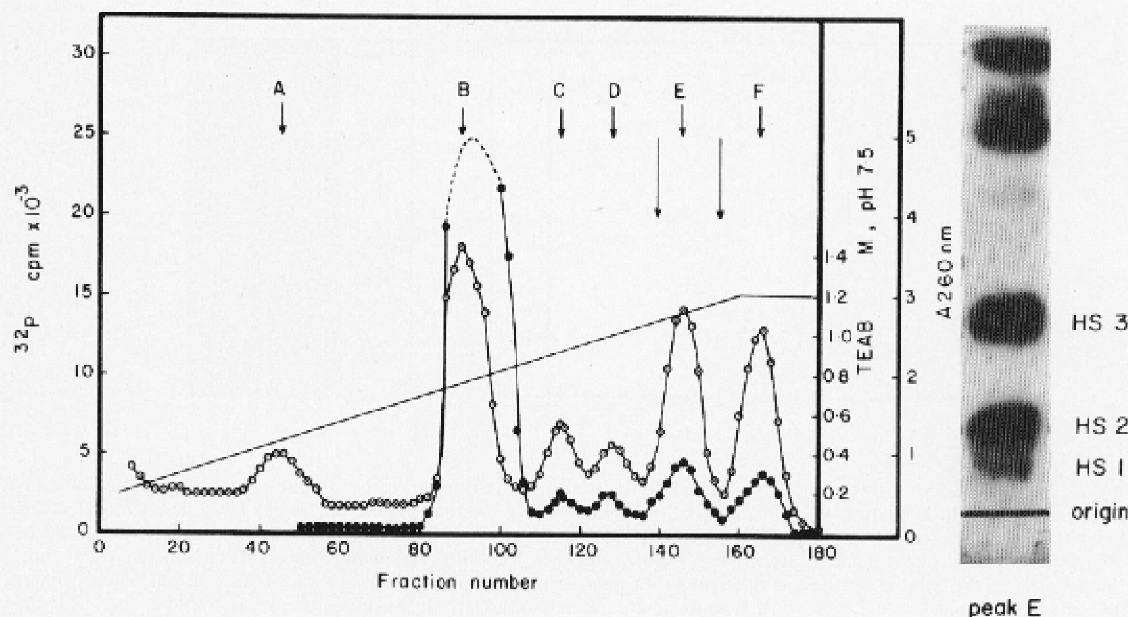

FIG. 1. Chromatography of ^{32}P-labelled formic-acid extract of 14 h-old *Achlya* mycelia in a column (3.0 × 45 cm) of DEAE-Sephadex preequilibrated with 0.2 *M* TEAB buffer pH 8. After the extract was loaded, the column was washed with 500 ml of 0.2 *M* TEAB buffer and then nucleotides were eluted with 500 ml of a linear (0.2–1.2 *M*) gradient of TEAB buffer. Four-millilitre fractions were collected. The A_{260} value (⊙) of each fraction was determined and the ^{32}P content (●) estimated by the Cerenkov technique. Also shown is an autoradiogram of the contents of peak E materials labelled with ^{32}P.

formic acid at 0°C was used to extract nucleotides from mycelia for 1 h. The acid was recovered by suction filtration, mycelia discarded, and the filtrate carefully adjusted to neutrality with ammonium hydroxide. The filtrate was then diluted 10-fold before fractionating on Sephadex A-25.

^{32}P-Labelled extract was isolated from small scale spinner flask cultures containing 500 ml growth medium and supplemented with 2–4 mCi (1 Ci = 37 GBq) [^{32}P]orthophosphate. The inoculum used for these cultures contained approximately 5×10^8 spores and the incubation time was 13–14 h at 28°C. Cells were collected and extracted with 2 ml of formic acid at 0°C. The ^{32}P-labelled extract was added to unlabelled extract from the large scale culture which was fractionated on Sephadex A-25 (Fig. 1). Peak E in Fig. 1 which eluted at 1.1 *M* TEAB buffer contained all three HS compounds as is seen in the autoradiograph of Fig. 1. Partially purified ^{32}P-labelled HS compounds (1) when chromatographed with unlabelled extract also eluted in peak E. Peak E material was evaporated to dryness, washed, resuspended in 0.05 *M* formate buffer pH 3.6, and chromatographed on Sephadex A-25 using a NaCl gradient at pH 3.6 (Fig. 2). Each peak was shown to contain a single component after PEI-cellulose chromatography. The autoradiographs of these chromatograms are also shown in Fig. 2 (right panel). HS3, HS2, and HS1 eluted at 0.28, 0.32, and 0.36 *M* NaCl respectively. The fractions within the boundaries of the arrows of Fig. 2 were pooled separately, concentrated, and desalted on Sephadex G-10.

Figure 3 shows the relative migratory positions of the three HS compounds with respect to GTP, ATP, ppGpp, and pppGpp after two-dimensional thin-layer chromatography on PEI-cellulose. The marker nucleotides were located by uv-light absorption but the HS compounds absorb very poorly on PEI-cellulose plates, possibly because of the high phosphate content of the elution buffer. It has been reported that increasing salt concentrations lowers the molar absorptivity of uracil-containing compounds (17).

Spectral Analysis of HS Compounds

Purified HS compounds were spectrally analysed between A_{220} and A_{300} nm at pH 1, 7, and 12 and the data are summarized in Table 1. The spectral data of adenosine and uridine (18) are also included. The HS compounds absorb maximally around 262 nm at each pH value with the wavelength of minimum absorption shifting as the pH changed from 1 to 12. This behaviour is characteristic of uracil and uracil-containing nucleotides. In addition, there was a decrease in the molar absorptivity of HS3 as the salt concentration (ionic strength) of the solution increased. HS3 lost 35% of its absorbance at 260 nm (pH 7) upon addition of 0.1 *M* NaCl while higher salt concentrations (2 *M* NaCl) did not cause any further decrease, a characteristic of uracil-containing nucleotides and polynucleotides as demonstrated by Simkins and Richards (17). They attributed this to base-stacking complications.

The uv-light absorbance of HS3 at 260 nm showed a hyperchromic effect of 10% during incubation with 0.33 *N* KOH at 37°C for 30 min. An increase of 20–25% in the uv-light absorbance at 260 nm was observed during

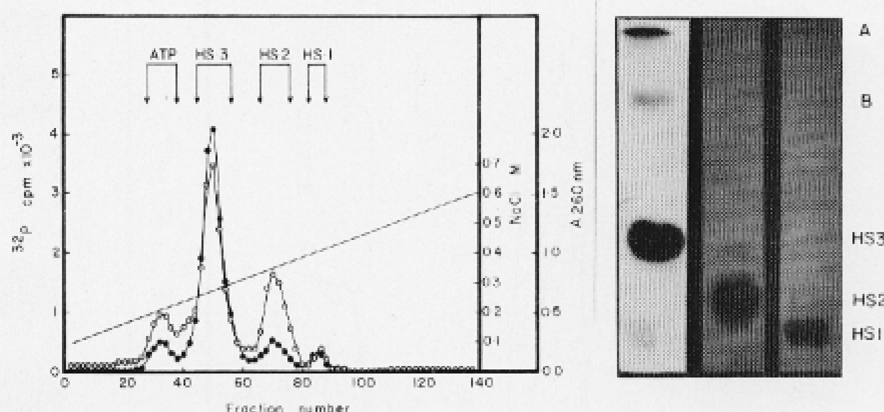

FIG. 2. Sephadex A-25 chromatography of the pooled fractions of peak E from Fig. 1. Material was loaded on a column (1.0 × 30 cm) which was then flushed with 250 ml of 0.1 *m* NaCl – 0.05 *M* sodium formate buffer pH 3.6. The nucleotides were eluted with a linear (0.1–0.6 *M*) gradient of NaCl in 0.05 *M* sodium formate pH 3.6. Four-millilitre fractions were collected and the A_{260} (⊙) and ^{32}P content (●) determined. Arrows indicate fractions that were pooled. Panel on the right shows autoradiograms of the contents of the last three peaks chromatographed on PEI-cellulose with solvent I. Breakdown products of HS3 arising from chromatography on PEI-cellulose are evident in the first of the three autoradiographic panels. They are marked as A and B and they migrate in areas corresponding to authentic UDP and ADP respectively.

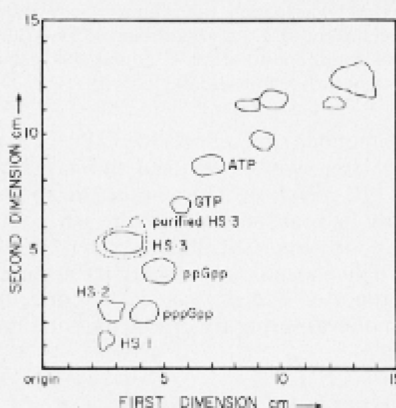

FIG. 3. Diagram of a two-dimensional PEI-cellulose chromatographic separation of formic-acid extract of 14-h *Achlya* cells grown in medium supplemented with $^{32}P_i$. Solvent II was used in the first dimension and solvent I in the second dimension.

treatment of HS3 with 1 *N* NCl for 30 min at 100°C, a treatment which degrades the molecule.

Characterization of HS3 Components

The following will deal mainly with a discussion of the data pertinent to HS3, but data on HS2 and HS1 are summarized in various tables.

Nucleobase Composition

^{32}P-Labelled HS3 was acid hydrolysed *in vacuo* for 1 h and 20 h in 1 *N* HCl at 105°C. The products were chromatographed on paper in solvent III. After a 1-h hydrolysis, HS3 was degraded into two uv-absorbing entities designated *a* and *c* in Table 2 (top). Component *a* had an R_f of 0.13 (adenine, R_f, 0.12) and *c* and R_f of 0.72 (UMP, R_f, 0.070). Component *a* did not contain ^{32}P label whereas *c* did. Both products were eluted from the paper, desalted on Sephadex G-10, and the uv spectra determined at pH 7.0. The spectra of *a* and *c* were similar to those of adenine and uridine monophosphate respectively (see Table 2 (bottom) for spectral ratios of authentic adenine and UMP). Molar absorptivities at 260 nm of 13.3 × 10^3 for adenine and 9.9 × 10^3 for UMP (18) were used to compute a mole ratio of 1:1 for the products *a* and *c*. After a 24-h acid hydrolysis of HS3, two products were observed. A uv-absorbing product designated *B* in Table 2 migrated with uridine (R_f, 0.56) while a fluorescent product migrated with an R_f value of 0.13.

The base constituents of ^{32}P-labelled HS3 were also determined after digestion of the molecule by a combination of enzymes. HS3 was digested with a mixture of nucleotide pyrophosphatase and bacterial alkaline phosphatase for 2 h (details given in the legend to Table 3). The hydrolysis products were separated chromatographically in two dimensions on thin-layer cellulose using solvents IV and V. Authentic adenosine and uridine were used as markers. As shown in Table 3, HS3 contained two uv-absorbing entities which comigrated in both dimensions with adenosine (product *a*) and uridine (product *b*). These two products were eluted from the cellulose with water and the carbohydrate content was determined as described below.

Amino Acid Analysis

After acid hydrolysis of HS3, analysis for amino acids was carried out. After two-dimensional thin-layer chromatography with solvents VI and VII, ninhydrin spray revealed a single, major, orange-red spot which was coincident with authentic glutamate (R_fs, 0.40 and 0.48). The presence of glutamate was confirmed and quantitated using a Beckman model 121 amino-acid analyser which showed that there was 1 mol of glutamate/mol of ribose (Table 4). Two other amino acids,

TABLE 1. Spectral data of HS compounds and of standards*

Compound	pH	λ_{max}	λ_{min}	Absorbance, 250:260	280:260	290:260
HS3	1	262	232	0.77	0.56	0.31
	7	260	232	0.84	0.45	0.16
	12	260	235	0.86	0.39	0.10
HS2	1	260	230	0.78	0.40	0.08
	7	260	230	0.76	0.39	0.06
	12	260	240	0.84	0.32	0.03
HS1	1	261	231	0.82	0.39	0.10
	7	260	233	0.86	0.37	0.10
	12	260	245	0.89	0.35	0.09
Adenosine*	1–7	257	230	0.84	0.21	0.03
	12	259	227	0.78	0.14	0.002
Uridine*	1–7	262	231	0.74	0.35	0.03
	12	262	242	0.83	0.29	0.02

*Data taken from Ref. 18.

TABLE 2. Chromatographic separation (top) and spectral analysis (bottom) of the uv-light absorbing products of HS3 after hydrolysis in 1 N HCl

Hydrolysis time, h	R_f values: HS3 products A	B	C	Standards Uridine	UMP	Adenine
Nil	—	—	—	0.56	0.70	0.12
1	0.13	—	0.72			
20	0.13	0.54	—			

Product	λ_{max}	λ_{min}	250:260	280:260	290:260	nmol*
A	258	228	0.90	0.17	0.03	5.3
C	263	230	0.86	0.36	0.02	5.4
Adenine	260	229	0.76	0.13	0.0005	
UMP	260	231	0.74	0.39	0.03	

*Extinction coefficients of 13.3×10^3 and 9.9×10^3 were used to calculate amounts of products A and C respectively. Data for controls and extinction coefficients were taken from Ref. 18.

TABLE 3. Chromatographic properties of the products derived from HS compounds degraded by bacterial alkaline phosphatase and nucleotide pyrophosphatase

Compound	Treatment	Chromatographic products, R_f: *a*	*b*
HS3	BAP* + NP*, 2 h, 37°C	0.63(0.30)	0.38(0.76)†
HS2	Same	—	0.38(0.76)
HS1	Same	—	0.38(0.76)
Uridine	None	—	0.38(0.74)
Adenosine	None	0.64(0.31)	—

*Enzymes used at 1 µg/OD of compound in 0.2 M Tris–HCl pH 7.5 and 0.02 M $MgCl_2$. BAP, bacterial alkaline phosphatase; NP, nucleotide pyrophosphatase.
†Figure outside of parenthesis describes the R_f value in the first dimension and that in parenthesis the R_f in the second dimension.

aspartic acid and glycine, were detected by the amino-acid analyser but they were present to the extent of only 9% and 11% respectively of the glutamate content. Either they were contaminants of the preparation or they were degradation products of adenine and uracil hydrolysis. Adenine was shown to yield glycine upon acid hydrolysis.

The glutamate from HS3 was hydrolysed to α-ketoglutarate by D-amino acid oxidase but not by L-amino acid oxidase indicating that the amino acid is the

TABLE 4. Stoichiometry of HS components

Compound	Ribose, nmol	PO_4^-, nmol	Base* nmol	Glu,† nmol	Ribose: base	Base: PO_4	Ribose: PO_4	Ribose: glu
HS3	10	76	18	9	1:1.8	1:4.2	1:7.6	1:1.1
HS2	8	75	17	—	1:2.1	1:4.3	1:9.3	—
HS1	6	72	12	—	1:2	1:6	1:12	—
ATP	8	25	8	—	1:1	1:3.1	1:3.1	—

*Total base was calculated from A_{260} values of acid-hydrolysed products with an extinction coefficient of 11.5×10^3 per base.
†Glu, glutamate.

TABLE 5. Carbohydrate analysis of HS3

Sugar	R_g solvent			Colour reaction with reagent					
	VIII	IX	X	M1	M2	M3	M4	M5	M6
X	2.25	1.7	3.0	+	−	+	+†	−	+
Y	1.3	1.15	1.1	+	−	+	+†	−	−
Ribose	3.3	1.8	4.5	+	+	+	+‡	+	−
Ribitol	2.2	1.7	3.1	+	−	+	+†	−	−
Mannitol	1.3	1.2	1.05	+	−	+	+†	−	−
Sorbitol	1.3	1.2	1.5	+	−	+	+†	−	−

*R_g indicates R_f values of sugars with respect to glucose as standard.
†Mauve colour appeared when respective sugars reacted with spray.
‡Indicates grey colour.

D optical isomer. Glutamate was not released from HS3 after heat treatment in water (20 h *in vacuo* at 105°C), periodate oxidation (19), or after digestion of HS3 with a mixture of bacterial alkaline phosphatase and nucleotide pyrophosphatase.

Carbohydrate Analysis

The purine-bound ribose content of HS3 was determined by orcinol. For the determination of pyrimidine-bound ribose, the sample was pretreated with 1% sodium amalgam for 20 h at room temperature (15). As summarized in Table 4, HS3 contained only one purine-bound ribose per molecule and no additional pyrimidine-bound ribose was detected. Either the ribose in the uridine residue was not being hydrolysed or ribose was not attached to the uracil. In order to differentiate between these two possibilities, the separated nucleosides produced by enzyme digestion (described above) after elution from the cellulose plate with water were analysed for ribose. The uridine nucleoside was found to contain pyrimidine-bound ribose equivalent in amount to the purine-bound ribose in adenosine. This suggests that the orcinol assay of HS3 estimated only the adenine-bound ribose and pretreatment of intact HS3 with sodium amalgam does not alter the stability of the C–N bond between the sugar and base in the uridine portion. HS3 must first be degraded to the component nucleosides before the uridine-bound ribose was estimable by the sodium-amalgam technique.

HS3 was acid hydrolysed and analysed for the presence of monosaccharides by paper chromatography. The results are shown in Table 5. In addition to the expected ribose, two other sugars designated X and Y were found. Monosaccharides X and Y were concluded to be sugar alcohols based on the following criteria. Neither sugar reacted with indicator sprays M2 or M5 suggesting that they were neither reducing sugars nor nonreducing sugars of the methyl pentose type. Both sugars reacted with indicator spray M1 very quickly suggesting that they were sugar alcohols and this was confirmed by their reactions with indicators M3 and M4. Sugar X was also found to be ninhydrin positive (indicator M6) indicating that it may contain an amino group. Sugar X migrated with an R_f value similar to that of ribitol in the three solvent systems (Table 5). Sugar Y migrated with an R_f value similar to those of mannitol and sorbitol in solvents VII and IX but was distinguished from sorbitol with solvent X. We have not been able to directly quantitate sugars X and Y. Indirect quantitation will be described subsequently.

Phosphate Analysis

Total phosphate was determined after ashing (11) and correlated with ribose and nucleobase content. The results presented in Table 4 indicate that HS3 has eight phosphates per molecule.

Partial Structure of HS3

HS3 was fragmented chemically and enzymatically. The fragments were isolated after column chromatography and analysed for their components and possible structures. Figure 4 gives a summary of the treatments and the results which are discussed in the following section.

(a) Alkali-hydrolysis Fragments

^{32}P-Labelled HS3 was incubated in 0.33 *N* KOH for 20 h at 37°C, and the mixture fractionated on Sephadex A-25 (Fig. 5). Two uv-light absorbing products each containing $^{32}P_i$ were detected and are designated as fragments A and B. The fractions of both peaks were collected and desalted. An aliquot of peak A material was chromato-

TABLE 6. Analyses of fragments produced by chemical and enzymatic digestion of HS3

Treatment of HS3	Products	R_f Solvent I*	R_f Solvent II†	Ribose	Base, nmol	PO_4	Ratios Ribose:PO_4	Ratios Ribose:base	Ratios Base:PO_4
KOH	A	0.57	0.27	0.9	0.8	1.6	1:1.9	1:1.1	1:2
	B	0.21	0.85	—	0.8	4.8	—	—	1:6
Nucleotide	C	0.68	0.20	1.5	1.5	1.4	1:1	1:1	1:0.94
Pyrophosphatase	D	0.78	0.95	—	—	1.5	—	—	—
	E	0.21	0.85	—	1.5	9.4	—	—	1:6.1

NOTE: The following are the R_f values for standard nucleotides and P_i: AMP (0.69*, 0.20†), ADP (0.60*, 0.27†), GTP (0.22*, 0.55†), UMP (0.76*, 0.56†), UTP (0.70*, 0.75†), and Pi (0.76*, 0.99†).
*Modified solvent I (see Materials and Methods).
†Solvent II.

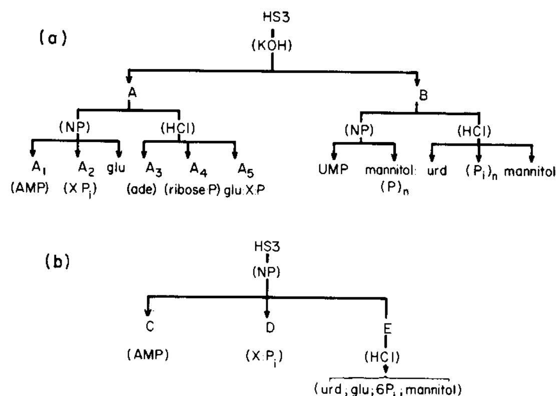

FIG. 4. Schematic diagrams of chemical and enzymic fragmentation of HS3. (*a*) Products after KOH, HCl, and nucleotide pyrophosphatase (NP) treatment; (*b*) products after NP treatment. A, B, C, D, and E as described in the text. glu, glutamate.

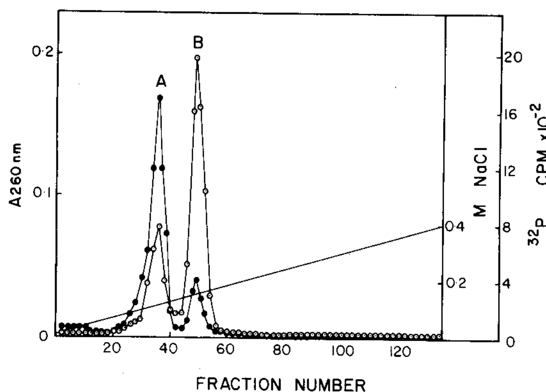

FIG. 5. Analysis of the products of KOH hydrolysis of HS3 on DEAE-Sephadex A-25 column. Ten A_{260} units of HS3 was incubated with 0.33 *N* KOH at 37°C for 20 h. The hydrolysate was diluted 10-fold with 0.05 *M* sodium formate and applied to a DEAE-Sephadex A-25 column (1 × 10 cm) preequilibrated with 0.01 *N* NaCl – 0.05 *M* sodium formate pH 3.6. The products were eluted using a 500-ml linear (0.01–0.4 *M*) gradient of NaCl in 0.05 *M* sodium formate pH 3.6; 4-ml fractions were collected and analysed for A_{260} (●) and ^{32}P (⊙) properties.

graphed one dimensionally on PEI-cellulose in modified solvent I (see Materials and Methods) and on cellulose paper in solvent III. The results are shown in Table 6. Material from peak A comigrated in both solvent systems with authentic ADP. The uv-light absorption spectrum of this material was similar to that of adenosine.

A sample of material from peak A (after concentration) was digested by alkaline phosphatase and nucleotide pyrophosphatase and the products chromatographed two dimensionally on thin-layer cellulose sheets. The uv-light absorbing product recovered comigrated with product *a* (see Table 3 for clarification) and adenosine. Quantitative analysis of the constituents of peak A gave the results (partly shown in Table 6) which are one adenine, one ribose, two phosphates, a polyhydric sugar X, and glutamate that could only be recovered after acid hydrolysis.

(b) Subfragments

Peak A material was degraded by nucleotide pyrophosphatase alone and the products separated on Sephadex A-25 (Fig. 6) into two components designated subfragments A_1 and A_2. Subfragment A_1 absorbed uv light and had ^{32}P in it. It also comigrated with authentic AMP with an R_f of 0.69 when chromatographed on PEI-cellulose with modified solvent I, and with an R_f of 0.20 when chromatographed on cellulose paper with solvent II. It was susceptible to periodate oxidation (19) suggesting that it might be 5′-AMP. Subfragment A_2 did not absorb uv light but had ^{32}P in it. It contained sugar X which was dissociated from the phosphate only after acid hydrolysis, suggesting covalent linkage. Both subfragments had equivalent ^{32}P radioactivities.

Neither subfragment A_1 nor subfragment A_2 contained glutamate tested for before and after acid hydrolysis. But the products of nucleotide pyrophosphatase hydrolysis (unfractionated) contained glutamate that was identified chromatographically after acid treatment. Thus, it would appear that glutamate was present in HS3 in a modified form (designated glutamate*) and was exposed only upon acid hydrolysis. Nucleotide pyrophosphatase therefore cleaved peak A material into AMP, 'X'MP and glutamate*.

A sample of material from peak A was subjected to limited acid hydrolysis and the products fractionated with Sephadex A-25 (Fig. 7) into three new subfragments called A_3, A_4, and A_5. Subfragment A_3 absorbed uv light but did not contain ^{32}P label. It was identified chromatographically as adenine. Subfragments A_4 and A_5 had equal amounts of ^{32}P. Analysis for carbohydrates showed that A_4 contained ribose and A_5 contained sugar

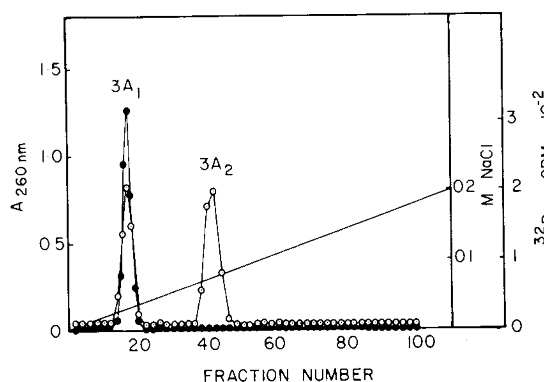

FIG. 6. Approximately 10 A_{260} units of fragment A was treated with nucleotide pyrophosphatase for 2 h at 37°C in 0.2 M Tris–HCl pH 7.5 and 0.02 M $MgCl_2$. Enzyme was used at 1 $\mu g/A_{260}$ unit. Mixture was diluted to 2 ml with 50 mM formate buffer pH 3.6 and loaded on Sephadex A-25 column (1 × 10 cm) preequilibrated with 0.01 M NaCl + 50 mM formate buffer pH 3.6. Products were eluted with a 200-ml linear (0.01–0.2 M) NaCl gradient in 50 mM formate buffer pH 3.6. Fractions (2 ml) were collected and analysed for A_{260} (●) and ^{32}P radioactivity (⊙). Fractions under peaks labelled A_1 and A_2 were collected and desalted on Sephadex G-10 prior to analysis.

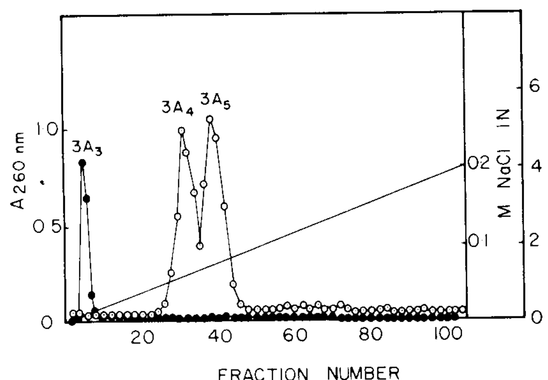

FIG. 7. Partial acid hydrolysis of fragment A. Approximately 5 A_{260} units was hydrolysed *in vacuo* in 1 N HCl at 105°C for 1 h. Acid was evaporated off and the residue dissolved in 50 mM formate pH 3.6 buffer and loaded on a Sephadex A-25 column (1 × 10 cm) preequilibrated with 0.01 M NaCl in 50 mM formate buffer pH 3.6. Products were eluted with a 200-ml linear (0.01–0.2 M) NaCl gradient in 50 mM formate pH 3.6 buffer. Fractions (2 ml) were collected and analysed for A_{260} (●) and ^{32}P radioactivity (⊙). Fractions under peaks A_3, A_4, and A_5 were collected and desalted before analysis.

X. After prolonged acid hydrolysis, the phosphates were removed from A_4 and A_5 suggesting covalent linkage. Ribose contamination in subfragment A_5 from A_4 was negligible. Glutamate was found to be associated with subfragment A_5. As subfragment A_5 contained only sugar X and phosphate, glutamate* must be linked to it either through the phosphate or sugar X itself. The arrangement of the components in fragment A would then be either glutamate*-XppA or Xp(glutamate*)pA.

Fragment B was analysed by similar methods for phosphate, carbohydrate, and nucleoside content. After digestion by nucleotide pyrophosphatase and alkaline phosphatase, the products of hydrolysis were chromatographed two dimensionally on thin-layer cellulose sheets. A single uv-light absorbing material that comigrated with uridine and product *b* (R_f of 0.38 in dimension 1; and 0.76 in dimension 2) described in Table 3 was obtained. As can be seen in Fig. 5, the A_{260} readings of fragments A and B were disproportionate. The absorbance for fragment B increased by 77.2% after desalting in Sephadex G-10. Therefore, the difference in A_{260} could be attributed to a lowering of the extinction coefficient of uracil in B because of salt effects (17). Hydrolysis of fragment B by 1 *n* HCl for 1 h at 105°C followed by the removal of the acid produced maximum absorption. Taking the extinction coeficient at 260 nm of 15.3 × 10^3 for fragment A and 9.9 × 10^3 for post acid-hydrolysed fragment B, a mole ratio of 1:1 was obtained for fragments A and B.

Fragment B did not react with orcinol directly, but after treatment with 1% sodium amalgam for 20 h, it showed 30–35% reactivity of the expected amount of ribose. The ^{32}P ratio between fragments A and B was 1:3. Since intact HS3 contain eight phosphates, fragment A therefore contains two phosphates and fragment B, six phosphates. Quantitation of phosphate by chemical analysis confirmed this ratio of 1:3 between A and B. Carbohydrate analysis of fragment B showed that it contained sugar Y which was identified as mannitol.

Fragment B was completely dephosphorylated in 2 h at 37°C using a mixture of bacterial alkaline phosphatase and nucleotide pyrophosphatase, yielding uridine. Therefore, the effects of each enzyme on fragment B were studied. Bacterial alkaline phosphatase was inactive on B. Nucleotide pyrophosphatase hydrolysed it very slowly. After a 4 h incubation, one product which absorbed uv light was recovered and it cochromatographed with UMP on paper. This product contained about 12% of the ^{32}P present in fragment B which corresponds to 0.75 (or about 1.0) phosphate. Fragment B was insensitive to periodate oxidation (19) indicating that no free adjacent hydroxyl groups exist on the constituent sugars, ribose, and mannitol. This enigmatic situation is considered fully in the Discussion.

Fragment B sugars were reactive to periodate after acid hydrolysis or after hydrolysis of the phosphates by alkaline phosphatase and nucleotide pyrophosphatase. Therefore, fragments A and B were acid hydrolysed and their carbohydrate content estimated by periodate oxidation. Fragment A with one ribose and one sugar X (assumed pentose) theoretically should consume 7 mol periodate. Fragment B, with one ribose and one mannitol sugar (Y), should consume 9 mol periodate. The theoretical ratio of periodate consumed between fragments A and B would then be 1:1.28. An experimental value of 1:1.2 was obtained suggesting that there is a molecule of each of sugar X and Y per HS3 molecule.

(c) Nucleotide-pyrophosphatase Hydrolysis Fragments

^{32}P-Labelled HS3 was hydrolysed with nucleotide pyrophosphatase and the products separated on Sephadex A-25 (Fig. 8). Three identifiable products desig-

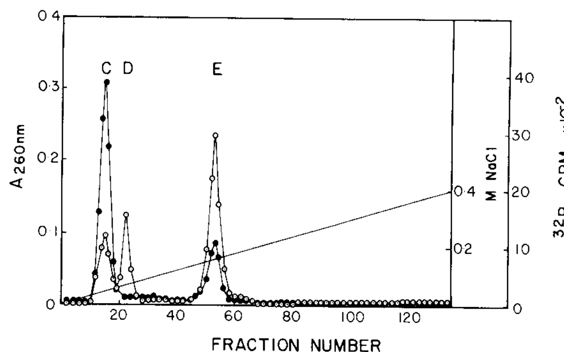

FIG. 8. Nucleotide pyrophosphatase digestion of HS3. Ten A_{260} units of HS3 was incubated with 0.1 *M* Tris–HCl pH 7.5, 0.02 *M* $MgCl_2$, and 1 mg/ml enzyme for 4 h at 37°C. After this, the mixture was diluted with 0.05 *M* sodium formate pH 3.6 buffer, loaded on a Sephadex A-25 column (1 × 10 cm), and eluted with a linear (0.01–0.4 *M*) NaCl gradient in 50 m*M* sodium formate pH 3.6. Fractions (4 ml) were collected and analysed for A_{260} (●) and ^{32}P radioactivity (⊙). Fractions under peaks C, D, and E were collected and desalted before analysis.

nated fragments C, D, and E were recovered. Only C and E contained uv-light absorbing materials whereas ^{32}P radioactivity was present in all three. Material from peak C migrated with AMP in both solvent systems (Table 6). The ribose to phosphate to adenine base ratio was computed as 1:1:1. Nucleoside analysis after enzymic digestion of peak C material with bacterial alkaline phosphatase indicated that it contained adenosine. Peak C material was susceptible to periodate oxidation, thereby confirming a 5′-phosphate linkage. Material from peak D migrated with P_i in both chromatographic systems (Table 6). Both ^{32}P radioactivity and colorimetric analysis for phosphate indicated that there were equivalent amounts of phosphate in fragments C and D. Carbohydrate analysis of the material in D showed that it contained sugar X.

Fragment E migrated slightly ahead of UTP during chromatography on cellulose paper with solvent III. On thin-layer PEI-cellulose plates, it migrated with GTP in one dimension with modified solvent I. Its composition was similar to that of fragment B except that it also contained glutamate.

Thus, glutamate seems to be associated with either the ADP – sugar X or UDP – mannitol tetraphosphate of HS3. The phosphate ratio among the fragments C, D, and E was 1:1:6. Digestion of HS3 for 6 h instead of 4 h with nucleotide pyrophosphatase resulted in the release of some UMP (data not shown) in addition to C, D, and E. Calculations based on uv-light absorbance showed a 40% loss of uv material from fragment E which was released as UMP.

Discussion

This communication has outlined the isolation, purification, and chemical, physical, and enzymic characterization (in part) of three unusual polyphosphorylated dinucleosides: HS3, HS2, and HS1. While much of the chemical components and organisation of HS3 has been determined, there are a few points of uncertainty that need to be discussed. But first, a summary of what is known about the structure of HS3 is pertinent.

HS3 is a dinucleoside polyphosphate that contains one purine (adenine) and one pyrimidine (uracil) base which exist as ADP and UDP respectively. A polyhydric alcohol related to ribitol (designated sugar X (and ninhydrin reactive)) is attached to ADP. A substituent which after acid hydrolysis becomes glutamate is also associated with the ADP – sugar X moiety. The UDP has a different polyhydric sugar alcohol, mannitol, and four phosphates attached to it. The ADP – sugar X and UDP – mannitol tetraphosphate moieties are covalently linked and the substituent that yields glutamate upon acid hydrolysis seems to be affiliated with both of them. It has not escaped our attention that the glutamate might be derived from a modified glutamine, thereby explaining the presence of a ninhydrin-sensitive group in sugar 'X'.

The uncertainities are as follows. To begin with, we have not yet resolved the manner in which the four phosphates associated with UDP and mannitol in fragment B (see Fig. 4*a*) are arranged. These phosphates are not removed as inorganic phosphate either with nucleotide pyrophosphatase or alkaline phosphatase. But a combination of the two enzymes does hydrolyse off all phosphates yielding uridine and mannitol. Nucleotide pyrophosphatase cleaved fragment B to UMP and mannitol polyphosphate indicating a pyrophosphate linkage between the two. Fragment B must therefore be a UDP – mannitol polyphosphate moiety. But as intact fragment B is insensitive to periodate oxidation, it is clear that the phosphates in the moiety must either be obstructing or blocking the adjacent hydroxyls in ribose and mannitol. Support for this concept comes from the observation that even after sodium-amalgam treatment, fragment B was only 35% reactive with orcinol.

Secondly, the mode of glutamate linkage and existence in HS3 is enigmatic for glutamate is only recovered after acid hydrolysis not after enzyme digestion. While glutamate appears to be covalently linked to subfragment A_5 (Figs. 4 and 7) which is a polyhydric sugar alcohol, the same sugar has a ninhydrin-reactive entity which reacts even after glutamate has been removed.

While HS2 and HS1 were described quantitatively for their ribose, nucleobase, and phosphate contents, we note that HS2 contains mannitol. We also note that HS2 and HS1 are chemically related based on the general products of acid hydrolysis (unpublished data). Work is currently underway to resolve their complete chemical compositions and structures.

A similar if not identical compound to fungal HS3 has been found in mammalian cells grown in cell culture (3). It was isolated from CHO cells starved of glutamine and shown to contain similar components as *Achlya* HS3 (4). Mammalian cell HS3, like *Achlya* HS3, has also been shown to inhibit the activities of DNA-dependent RNA polymerases and ribonucleotide reductases of *Achlya* and CHO cells in a like manner. A physiological role for HS3 in eukaryotes is worth investigating.

Acknowledgement

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