ENZYMATIC FORMATION OF TDP-3-ACETAMIDO-3,6-DIDEOXY HEXOSE

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Upon examination of various nucleotide-linked sugars as glycosyl donors in the cell free biosynthesis of an exopolysaccharide produced by Xanthomonas campestris (1), TDP-glucose was found to give rise to a series of unusual amino sugars. One of the members of this group has been isolated in apparently pure form and identified as TDP-3-acetamido-3,6-dideoxy hexose. The present communication provides the initial evidence in support of this identification.

The organism, Xanthomonas campestris [2] was grown on a malt-yeast extract medium (2) and harvested after shaking for 18-24 hours at room temperature. The cells were washed twice with distilled water, suspended in 6.5 volumes of 0.05 M $(NH_4)_2HPO_4$ containing 0.01 M glutathione, and disrupted for a period of 10 minutes in a 10 kc. Raytheon Sonic Oscillator.

The reaction mixture contained 100 μmoles of TDP-glucose-C^{14} (specific activity, 2×10^4 cpm/μmole, uniformly labelled in the glucose moiety) and 100 ml of the crude sonicate. After incubation for 1 hour at room temperature, the reaction mixture was immersed in a boiling water bath for 3 minutes followed by rapid chilling in ice and adjustment of the pH to 3.5. The resulting precipitate was removed by centrifugation and 3.0 gm of acid washed Norite were added. After several washes with cold water, the radioactive nucleotides were eluted from the charcoal with 50% ethanol containing 0.1% NH_4OH. Approximately 50% of the total radioactivity was recovered in this fraction. The eluate was

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[2] A culture of the organism was kindly made available to us by Dr. Allene Jeanes and Dr. R. F. Anderson of the Northern Regional Research Laboratory, Peoria, Illinois.

concentrated and subjected to descending chromatography in ethanol: 1 M ammonium acetate (7:3) overnight. Two distinctly separable radioactive peaks were obtained which were eluted from the paper and rechromatographed employing an isobutyric acid: 1 M NH_4OH (10:6) solvent system.

As summarized in Table I, 4 nucleotide-linked radioactive compounds were obtained, all of which showed absorption spectra characteristic for thymidine. The present communication will restrict itself to documentation on the basic structure of Peak II although a considerable body of evidence is available to support the conclusion that Peak I contains TDP-rhamnose plus two TDP-amino sugars structurally similar to Peak II.

TABLE I

	95% Ethanol: 1 M ammonium acetate (7:3)	Isobutyric acid: 1 M NH_4OH (10:6)
Peak I	1.19	0.50; 0.67; 1.0
Peak II	1.47	0.81

The figures refer to the migration of the unknowns in relation to the migration of thymidylic acid.

Peak II was recovered from the isobutyric acid chromatogram and further purified by adsorption onto and elution from Norite. This material proved to be homogeneous in a variety of chromatographic and electrophoretic systems. An additional parameter of purity was the demonstration that the specific activity of this material, based upon the ϵ_M for thymidine of 9.6×10^3 at 267 mμ, was identical with that of the starting TDP-glucose, i.e., 2×10^4 cpm/μmole. Table II summarizes the analytical data obtained with the intact nucleotide.

The effect of acid hydrolysis on Peak II under mild and drastic conditions is illustrated in Table III. At pH 2, a single neutral compound was liberated from the nucleotide whereas upon prolonged hydrolysis in 2 N HCl, a positively

TABLE II

Compound	Periodate consumption *	Phosphorus Assay		λ_{MAX}	λ_{MIN}	$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$
		Acid labile *	Total *				
TDP-Glucose	2.0	1.17	1.93	267	234	0.64	0.72
Peak II	1.92	1.18	1.91	267	234	0.66	0.76

* The figures represent the calculated molar ratios based upon an ϵ_M of 9.6 x 10^3 at 267 mμ and pH 7. Periodate oxidation was allowed to continue for 44 hours in the dark at pH 8.0 and measured by titration of the excess arsenite with 0.005 N I_2. Acid hydrolysis was carried out in 1 N HCl at 100° for 10 mins.

charged product appeared, exhibiting a marked cathodal migration in an electrophoretic field at pH 4.0. Re-acetylation of the latter compound with acetic anhydride in $NaHCO_3$ buffer yielded a neutral product which co-chromatographed with the original, mildly hydrolyzed material. These data strongly suggest that Peak II is an N-acetylated amino sugar.

TABLE III

Conditions	Isoamyl acetate: acetic acid: water (30:30:10)	Ethyl acetate: pyridine: water (72:20:23)	High volt electrophoresis pH 4.0
Peak II hydrolyzed in 0.01 N HCl for 10 minutes at 100°	1.21	0.73	Neutral
Peak II hydrolyzed in 2 N HCl for 2 hours at 100°	0.33	0.095	Migrated to the cathode
Re-acetylated Peak II after hydrolysis in 2 N HCl	1.21	0.73	Neutral

The figures refer to the migration of the unknown in relation to the migration of L-rhamnose. The location of the above radioactive compounds was determined with an automatic chromatogram scanner.

Periodate degradation of the material obtained by hydrolysis in 2 N HCl resulted in the formation of 1 molar equivalent of acetaldehyde (3) based upon the values obtained by the simultaneous degradation of authentic L-rhamnose. Quantitative titration of periodate consumption revealed 3.93 moles of periodate utilized per mole of the acid hydrolyzed product, a finding in accord with the postulation of a 6-deoxy hexose.

Acid hydrolyzed Peak II failed to react as a 2-amino sugar when assayed by the Elson-Morgan (4) or Morgan-Elson (5) reactions and was readily distinguished from glucosamine, galactosamine, mannosamine, fucosamine, and 4-amino-4-,6--dideoxy hexose [3/] by paper and column chromatography.

In an experiment designed to locate the position of the amino group on the hexose structure, 1.0 μmole of Peak II was hydrolyzed in 0.01 N HCl for 10 minutes at 100°. Upon cooling, the liberated sugar was converted to a sugar acid by hypoiodite oxidation. The latter compound, in turn, was subjected to periodate oxidation, pH 8.0, for 2 hours in the dark and the aldehyde products of this reaction again oxidized with hypoiodite. After removal of excess I_2, the final reaction product was hydrolyzed for 2 hours at 100° in 2 N HCl in order to remove the N-acetyl substituent, applied to Whatman #1 paper and developed overnight in butanol:acetic acid:water (100:22:50).

Two radioactive peaks were recovered, one of which co-chromatographed with authentic β-hydroxy aspartic acid and exhibited the same initial green-brown color upon staining with ninhydrin. A second, smaller radioactive peak failed to react with ninhydrin and presumably represented incompletely degraded amino-hexonic acid.

After elution from paper, the over-all recovery of the ninhydrin-positive material represented 40-45% of the maximum theoretical conversion. This product was found to co-chromatograph with β-hydroxy aspartic acid in several chromatographic systems which clearly resolved it from threonine, allothreonine, serine,

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[3/] A sample of crystalline 4-amino-4,6-dideoxy hexose was generously provided by Dr. Robert Wheat of Duke University.

alanine, and aspartic acid. In order to prove the identity of the isolated amino acid and simultaneously to determine its enantiomorphic configuration, 2,000 cpm of the radioactive product were added to 20 mg of authentic erythro--D,L-β-hydroxy aspartic acid and a similar amount of radioactivity was added to 20 mg of authentic threo-D,L-β-hydroxy aspartic acid. After 5 recrystallizations, the radioactivity remained constant with the threo isomer and diminished steadily to zero in the presence of the erythro isomer (Table IV).

TABLE IV

Number of recrystallizations	Recrystallized in presence of authentic erythro-D,L-β-hydroxy aspartic acid	Recrystallized in presence of authentic threo-D,L-β-hydroxy aspartic acid
	cpm/mg *	cpm/mg *
1	55	89
2	37	89
3	6	93
4	2	82
5	0	91

* Counts were corrected for self-absorption.

The identification of the amino acid degradation product as β-hydroxy aspartic acid establishes the location of the amino group on carbon-3 of the hexose chain. It is significant to point out that an analogous TDP-linked 4-amino-4,6-dideoxy hexose has recently been described in which threonine was obtained by a similar series of reactions (6).

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