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Isolation of N^6-(Aminoacyl)adenosine from Yeast Ribonucleic Acid*

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A group of compounds with the general structure, N^6-(aminoacyl)adenosine, I, has been isolated from the soluble RNA fraction of yeast. The aminoacyladenine bond is stable to 0.5 N hydrochloric acid at 100° for 2 hours, to alkaline buffer (*p*H 10.5) at room temperature for 24 hours, and to 2.0 M hydroxylamine (*p*H 7.0) at room temperature for 3 hours. The following amino acids have been found in this group: alanine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, and valine, of which aspartic acid, glycine, threonine, and valine predominate. Although the majority of these compounds appear to consist of adenosine and a single amino acid, the possibility exists that some of these compounds consist of adenosine and a polypeptide chain.

Several reports have appeared in the literature which describe the isolation of polypeptides and amino acids linked covalently to nucleotides.[1] These compounds have been found in both the acid-soluble extracts and the RNA of a number of tissues and, according to the nature of the covalent bond between the amino acid and nucleotide, may be classified into three groups. These classifications are as follows:

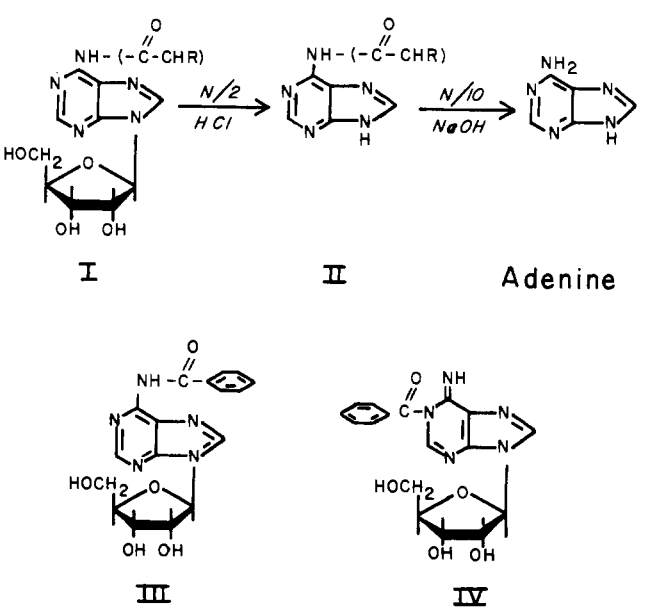
Phosphoanhydride Linkage.—Polypeptidyl-nucleotides, in which the carboxyl group of the terminal amino acid of the polypeptide chain is attached to a phosphate residue of the nucleotide in an anhydride bond, have been isolated from yeast (Harris and Wiseman, 1962; Schuurs and Koningsberger, 1960).

Ester Linkage.—Amino acids are attached to transfer RNA by esterification of the 2′- or 3′-hydroxyl of the terminal adenosine unit (Zachau *et al.*, 1958; Preiss *et al.*, 1959). Habermann (1959) and De Kleot *et al.* (1960) have shown that yeast and mammalian RNA contain firmly bound polypeptides. They suggest that as in the above case the carboxyl end of the polypeptide chain is linked to the 2′- or 3′-hydroxyl group of the nucleoside residue. Harris and Wiseman (1962) have isolated small oligonucleotides from yeast in which polypeptides are similarly bound.

Phosphoramide Linkage.—Potter and Dounce (1956) isolated nucleotide fractions from the RNA of yeast, calf pancreas, and rabbit liver which contained firmly bound amino acids and short polypeptides. On the basis of their evidence they suggested that these compounds are bonded between the phosphate residue of the nucleotide and an available amino group of the peptide chain. A more recent report (Bogdanov *et al.*, 1962) presents similar evidence for the existence of such nucleotide-peptide complexes. A considerable body of information has thus accumulated concerning the occurrence of amino acid- and peptide-nucleotides in nature. The purpose of the present paper is to report the isolation of a hitherto unrecorded type of aminoacyl-nucleoside which, on the basis of chemical evidence, has been assigned the structure, N^6-(aminoacyl)adenosine, I.

EXPERIMENTAL

Yeast s-RNA (22 g, 70% pure) was prepared for us by General Biochemicals according to the method



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[1] Many of the earlier references have been summarized in a paper by Wilken and Hansen (1961).

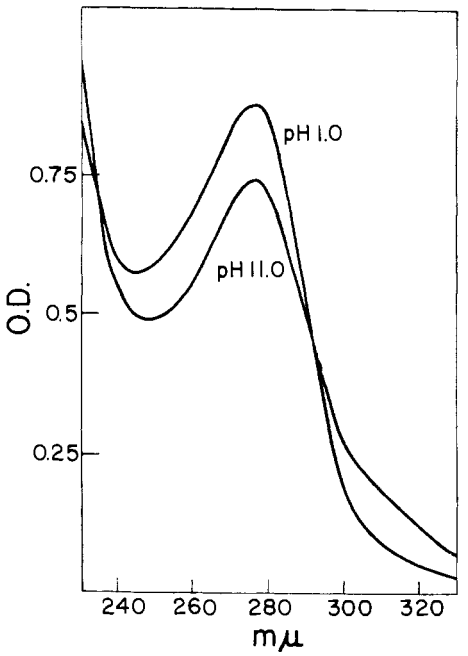

FIG. 1.—Presumed N^6-(aminoacyl)adenine–ultraviolet-absorption spectra of compound obtained by hydrolysis of N^6-(aminoacyl)adenosine in 0.5 N hydrochloric acid for 30 minutes at 100°.

of Holley *et al.* (1961). The sample was dissolved in 1.7 liters of distilled water and dialyzed against two changes of cold distilled water over a 24-hour period. There was no loss of ultraviolet-absorbing material, as measured by the total optical density of the sample. To the solution was added 1.7 cc of 1 M $MgSO_4$ and the RNA was digested with 2.0 g of *Crotalus adamanteus* venom (Ross Allen Reptile Farm) and 45 mg of purified bacterial alkaline phosphatase (Worthington Biochemical Corp.). Toluene was added to the digestion solution to eliminate bacterial contamination. The *p*H was maintained at 8.6 throughout the digestion by addition of 1 N sodium hydroxide. After 7 hours, 1 cc of 1 M magnesium sulfate, 25 mg of the alkaline phosphatase, and 700 mg of venom were added, and digestion was continued for 16 hours. (Analysis for inorganic phosphorus gave a result of 1.37 g; expected, 1.36 g.) The solution was concentrated to 1 liter and heated to 60° for 30 minutes, and the *p*H was adjusted to 6.0. After being chilled in a refrigerator overnight, the solution was centrifuged at 15,000 × *g* for 1 hour. The clear supernatant was lyophilized to yield 21.5 g of material of which the free nucleosides represented 11.5 g.

Column Procedure.—The lyophilized sample was divided into four equal lots. Each lot was fractionated by means of partition chromatography into six ribonucleoside fractions by the method previously described (Hall, 1963). The column used for each separation measured <5 × <121 cm (2 in. × 4 ft) and was charged with 770 g of Celite-545–Microcel-E[2] (9:1). The solvent systems used previously were slightly modified as follows: Solvent 1, ethyl acetate–2-ethoxyethanol–water (4:1:2). Solvent 2, ethyl acetate–1-butanol–ligroin–water (1:2:1:1).

Isolation of N^6-(Aminoacyl)adenosine.—The fractions containing cytidine were combined and rechromatographed on a partition column packed with 150 g of Celite-545 according to the basic procedure of Hall (1962). The solvent system, ethyl acetate–1-butanol–water (1:1:1) was used. A small fraction of ultraviolet-absorbing material was eluted followed by a large fraction corresponding to a single ultraviolet-absorbing peak. The amino acid–adenosine compounds were located in the fractions corresponding to the latter two-thirds of the large peak. This fraction was concentrated to a small volume and streaked across Whatman 3 MM paper (several sheets equivalent to >548 lin cm [216 lin in.]). The sheets were developed for 24 hours in the solvent system E. Several bands of ultraviolet-absorbing material were observed of which the fastest-moving band consisted solely of the amino acid–adenosine compounds. This band was eluted with water and the combined eluates were concentrated to 3.75 cc. This solution represented the working solution for subsequent experiments. The total amount of amino acid–adenosine compounds was estimated to be approximately 15 μmoles, based on the amount of adenosine, measured spectrophotometrically, yield 0.034% (based on total nucleosides in original digest).

Characterization of N^6-(Aminoacyl)adenosine (Compound I).—The product in the stock solution moved as a single spot on paper in several solvent systems (Table I). For the purpose of simplifying subsequent discussion of this product it is described as a single compound. Treatment of compound I with 0.1 N sodium hydroxide for 20 minutes at 100° yielded a new compound which gave ultraviolet-absorption curves identical with those of adenosine at all *p*H values and moved with adenosine on paper strips in several solvent systems (see Table I). Treatment of compound I with 0.5 N hydrochloric acid for 2 hours at 100° resulted in hydrolysis to a new compound, II, which on paper strips had the R_F values shown in Table I and gave the ultraviolet-absorption spectra shown in Figure 1. The spectra of this compound are similar to those recorded for the glycyladenine, synthesized by Brink and Schein (1963), which is considered to be N^6-glycyladenine. When the presumed N^6-(aminoacyl)adenine, compound II, was boiled in 0.1 N sodium hydroxide solution for 30 minutes, adenine was obtained. Its identification was based on the fact that the product of hydrolysis gave ultraviolet-absorption spectra identical with that

TABLE I
PAPER CHROMATOGRAPHY

Compound	Solvent System (R_F Values) A	B	C	D[a]	E
N^6-(Aminoacyl)-adenosine (I)	0.23	0.64	0.36	0.33	0.51
Adenosine obtained from compound I by alkaline hydrolysis	0.34	0.58	0.36	1.00	0.45
Adenosine	0.34	0.58	0.36	1.00	0.45
Adenine obtained from compound I by acid and alkaline hydrolysis	0.52	0.62	0.33	1.5	0.42
Adenine	0.52	0.62	0.33	1.5	0.42
N^6-(Aminoacyl)-adenine	0.34	0.62	0.33		0.45

[a] Value relative to adenosine. Solvents: A, 1-butanol–water–concd ammonium hydroxide (86:14:5); B, 2-propanol–1% ammonium sulfate solution (2:1); C, 2-propanol–concd hydrochloric acid–water (680:170:144); D, ethyl acetate–1-propanol–water (4:1:2); E, 2-propanol–water–concd ammonium hydroxide (7:2:1).

[2] Trade names of the Johns-Manville Co. for diatomaceous earth.

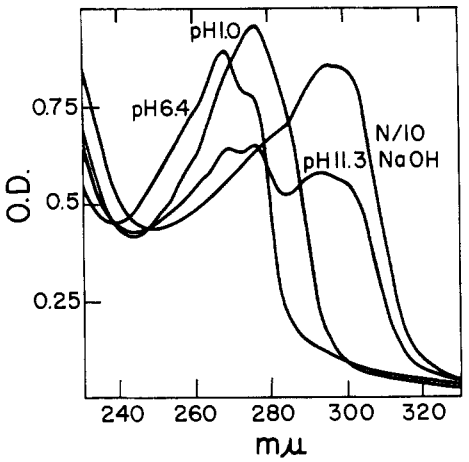

FIG. 2.—N^6-(Aminoacyl)adenosine–ultraviolet-absorption spectra of a 0.041 mM solution (concentration estimated on basis of adenosine content).

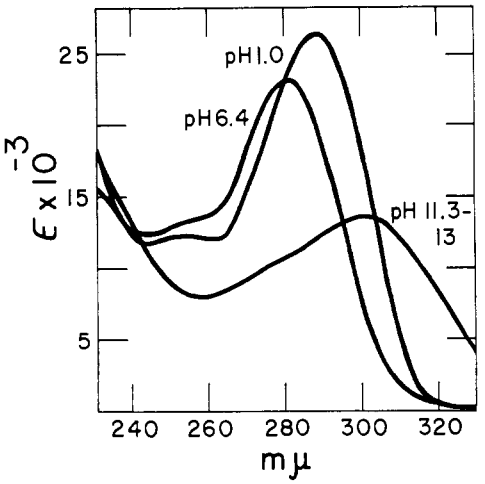

FIG. 3.—N^6-Benzoyladenosine–ultraviolet-absorption spectra of a 0.039 mM solution.

of adenine in 0.1 N hydrochloric acid and 0.1 N sodium hydroxide, and further, its movement on paper strips in five solvent systems corresponded with that of adenine (Table I).

Further evidence that the amino acid of the isolated nucleoside is attached to the base portion is furnished by the ultraviolet-absorption spectra (Fig. 2). These spectra suggested that the linkage is through an acyl bond, and for purposes of comparison a model acyladenosine, N^6-benzoyladenosine (compound III), was prepared as follows. Adenosine (5.34 g, 20 mmoles) and freshly distilled benzoyl chloride (28 cc, 240 mmoles) were dissolved in 120 cc of pyridine. The solution was allowed to stand at room temperature for 22 hours after which time 15 cc of methanol was added. One hour later the pyridine hydrochloride was filtered off and the filtrate was evaporated to a gum. The residue was dissolved in chloroform and washed three times with water. The chloroform solution was dried over sodium sulfate and evaporated to dryness. The residue was dissolved in 200 cc of pyridine. To the pyridine solution was added 200 cc of 2 N sodium hydroxide. The solution was shaken for 90 minutes, then sufficient Dowex-50 [H^+] resin was added to remove the sodium ions. The residue was washed with a little ethanol, then with water. The solution was extracted four times with ether and then set aside at 4° for several hours. N^6-Benzoyladenosine crystallized out of the solution, wt 5.8 g, 80%. The crude precipitate was crystallized from 200 cc of methanol-water (2:1), mp 152–155° capillary-uncorrected, 4.3 g. Two more crystallizations raised the melting point to 159–164°. Repeated recrystallization did not sharpen the melting point.

Anal. Calcd. for $C_{17}H_{17}N_5O_5 \cdot H_2O$: C, 52.44; H, 4.92; N, 17.99. Found: C, 51.94; H, 4.95; N, 18.05. The ultraviolet-absorption spectra are shown in Figure 3.

The initial reaction product is most likely a pentabenzoyl compound in which two of the benzoyl groups are attached to the adenine residue (Smith *et al.*, 1962). Controlled alkaline hydrolysis removes the three *O*-acyl groups and one of the adenine acyl groups. The remaining benzoyl group presumably is in the N^6 position (Ralph and Khorana, 1961) although an alternative N^1 structure (IV) cannot be excluded.

Additional Properties of N^6-(Aminoacyl)adenosine.—This compound is stable in a buffer at *p*H 8.6 at 37° for 24 hours. It did not hydrolyze when left in a buffer at *p*H 10.5 at room temperature for 24 hours. It undergoes hydrolysis in 0.1 N sodium hydroxide at 37° as shown in Figure 4. The shape of the curve suggests that there is a mixture of compounds hydrolyzing at various rates.

Compound I is not attacked by hydroxylamine (2 M solution, *p*H 7.0, 3 hours at 37°), which is in contrast to nucleosides with 2′- (or 3′-) linked peptidyl bonds (Harris and Wiseman, 1962).

The presence of free *cis*-hydroxyl groups in compound I is demonstrated by the observation that the electrophoretic mobility of this compound is substantially affected by the presence of borate ion in the buffer (Table II).

TABLE II
ELECTROPHORESIS[a]

Compound	Distance Moved from Origin: 0.05 M Glycine Buffer *p*H 9.2 (cm)	Distance Moved from Origin: 0.05 M Glycine, 0.05 M Borate Buffer *p*H 9.2 (cm)
N^6-(Aminoacyl)-adenosine	−4.0	+8.0
Adenosine	−3.5	+8.0

[a] Experiment carried out for 4 hours at 22 v/cm.

Amino Acid Analysis.—(a) Paper Chromatography.—To 100 μl of stock solution was added 100 μl of concentrated hydrochloric acid and the solution was heated at 100° for 20 hours. The hydrolysate was dried over potassium hydroxide and the residue was subjected to two-dimensional chromatography-electrophoresis. *Direction 1:* Chromatography in 1-butanol–acetic acid–water (4:1:5). *Direction 2:* Electrophoresis in Gilson Electrophorator, in 4% formic acid; 2000 v for 67 minutes. The positions of the ninhydrin spots were compared with those of known amino acids on a standard chromatogram, which indicated the presence of alanine, aspartic acid, glutamic acid, glycine, leucine and/or isoleucine, serine, threonine, valine, and one unidentified spot. These data are confirmed by the results obtained from the amino acid analyzer (see Table III).

Another portion of the stock solution was heated in the presence of sodium hydroxide (final concentration 0.05 N) at 100° for 30 minutes. This treatment is sufficient to completely hydrolyze the aminoacyl-

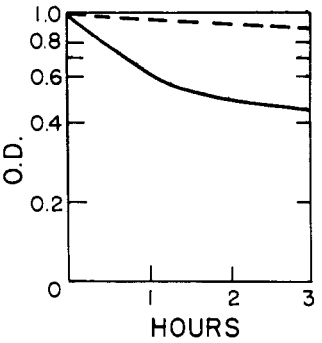

FIG. 4.—Rate of hydrolysis of N^6-(aminoacyl)adenosine and N^6-benzoyladenosine as indicated by decrease in OD at 298 mμ. Sample dissolved in 0.1 N sodium hydroxide and incubated at 37° in heated compartment of spectrophotometer. N^6-Benzoyladenosine, ---------; N^6-(aminoacyl)adenosine, ———.

TABLE III
AMINO ACIDS AS DETERMINED BY QUANTITATIVE ION-EXCHANGE CHROMATOGRAPHY[a]

	Amount (μmoles)	Ratio
Alanine	0.097	3
Aspartic acid	0.199	7
Glutamic acid	0.061	2
Glycine	0.782	26
Isoleucine	0.082	3
Leucine	0.029	1
Methionine	0.027	1
Phenylalanine	0.027	1
Proline	0.018	0.5
Serine	0.096	3
Threonine	0.429	15
Valine	0.843	28
Total	2.7	

[a] The total amount of N^6-(aminoacyl)adenosine hydrolyzed was estimated spectrophotometrically to be about 4.0 μmoles.

adenosine bond. Chromatographic analyses conducted as above indicated the presence of alanine, aspartic acid, glycine, threonine, and valine.

(b) Quantitative Column Chromatography.—To 1 cc (4.0 μmoles) of stock solution was added 1 cc of concentrated hydrochloric acid and the mixture was heated at 100° for 20 hours. The hydrolysate was dried over potassium hydroxide and the residue was subjected to quantitative ion-exchange chromatography according to the method of Spackman *et al.* (1958). The results are tabulated in Table III.

DISCUSSION

The substance isolated and described as N^6-(aminoacyl)adenosine consists of a group of compounds, each containing adenosine and an amino acid. The data indicate that the ratio of amino acid to adenosine is close to one. This conclusion is based on the observation that mild alkaline hydrolysis which would break only the acyladenosine bond releases, as free amino acids, alanine, aspartic acid, glycine, threonine, and valine. These five amino acids comprise 87% of the total amino acid content according to the data obtained by quantitative ion-exchange column chromatography. The remaining 13% of the amino acid content is made up of several additional amino acids, most of which were detected by paper chromatography-electrophoresis of the 6 N hydrochloric acid digest of the N^6-(aminoacyl)adenosines. The possibility thus arises that a small portion of the amino acids may have been present originally as part of polypeptide chains. The total recovery of amino acids from the ion-exchange column considering experimental losses also supports the conclusion that the majority of the isolated adenosine–amino acid compounds consist of adenosine and one amino acid. Further investigation of the amino acid content of this group of compounds will have to wait until additional amounts can be isolated.

The nature of the amino acid–adenosine linkage is clearly established by the data. Evidence that the amino acid is attached to the adenine residue of the nucleoside stems from the fact that mild acid hydrolysis of compound I removes the sugar residue, leaving an amino acid–adenine compound (compound II). The distinctive ultraviolet-absorption spectra (Fig. 2) of the isolated compound, I, further support this conclusion and indicate the acyl nature of the bond. Attention is drawn to the shifts of the absorption maxima to longer wavelengths in both acidic and alkaline solution which resemble closely the pattern of N^6-benzoyladenosine. The inflections in the spectra of the aminoacyladenosines are not observed in the spectra of N^6-benzoyladenosine, but this would be due to the fact that the spectra in Figure 2 are actually a composite of several compounds, or could be owing to the influence of the α-amino groups. An alternative form of binding, exemplified by adenylosuccinic acid, in which the amino group of adenosine is common with the amino group of the amino acid, is ruled out on the basis of the vastly different ultraviolet-absorption spectra (i.e., λ_{max} *p*H 1.0, 267; λ_{max} *p*H 12, 270; Carter and Cohen, 1955).

It should be pointed out that the model compound, N^6-benzoyladenosine, has not been prepared by an unambiguous method. Ralph and Khorana (1961) investigated the structure of the benzoyladenylic acid they prepared by a similar method and concluded that attachment at the N^6 poisition is more reasonable than the alternative attachment at the N^1 position. Therefore, unless the properties of synthesized model compounds should suggest otherwise, the amino acids of the isolated compounds are considered to be attached at N^6 of adenosine. This does not exclude the possibility that the natural compounds have the amino acid linkage at the N^1 position and that rearrangement to the N^6 position occurs during isolation analogous to the rearrangement of N^1-methyladenosine to N^6-methyladenosine (Dunn, 1961). Shabarova *et al.* (1959) have synthesized a compound, claimed to be N^6-(*N*-phthaloylglycyl)adenosine; but since they did not characterize this compound, it is not possible to compare the properties of the natural product with those of their synthetic product.

The assumption has been made that this group of compounds originated in the yeast; but since snake venom and bacterial alkaline phosphatase were added during the isolation procedure, the possibility exists that these other natural products represent the source. In order to eliminate this possibility 2.0 g of snake venom and 50 mg of bacterial alkaline phosphatase were combined and incubated under the conditions used in the RNA hydrolysis. Examination of the incubated mixture on a partition column under analogous conditions used for isolation of N^6-(aminoacyl)-adenosine revealed no such compound. Therefore it is most probable that the source of the compounds is yeast.

The degree of purification of the s-RNA used in this work suggests that the s-RNA, rather than a small oligonucleotide isolated concomitantly, represents the

source of N^6-(aminoacyl)adenosine. There have been previous indications that yeast RNA contains amino acids which are relatively firmly bound. Ingram and Sullivan (1962) showed that yeast s-RNA contains amino acids, at a level of about one per molecule of s-RNA, which are quite distinct from the amino acids involved in the s-RNA–amino acid exchange. It is possible that the amino acid–RNA complexes described by these workers as well as by others (Ishihara, 1960; Jonsen *et al.*, 1959) are in actual fact the source of the N^6-(aminoacyl)adenosine group described in this paper. The small amount of the N^6-(aminoacyl)adenosines isolated in the present work is not necessarily a true representation of the levels found in nature, as undoubtedly during the isolation procedure some degradation and fractionation of this group occurred.

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Studies on UDPG: α-1,4-Glucan α-4-Glucosyltransferase. VI. Specificity and Structural Requirements for the Activator of the *D* Form of the Dog Muscle Enzyme*

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Received January 8, 1964

The present paper of this series deals with the specificity of the activator molecule and demonstrates some of the requirements that are necessary for activation. Of over thirty compounds tested, glucose-6-phosphate was the most potent activator. Important structural features of the molecule include the hydroxyl groups at carbons 2, 4, and 3, the phosphate attached at carbon 6, and a pyranose ring structure.

The enzyme UDPG: α-1,4-glucan α-4-glucosyltransferase (transferase)[1] catalyzes the transfer of the glucosyl residue from UDPG into glycogen. Leloir *et al.* (1959) reported that transferase from rat skeletal muscle was activated by glucose-6-phosphate, glucosamine-6-phosphate, galactose-6-phosphate, and fructose-6-phosphate. The last three compounds activated to a lesser extent than glucose-6-phosphate (Traut, 1962). Leloir and Goldemberg (1960) demonstrated that the enzyme prepared from liver was also activated by these sugar phosphates (Steiner *et al.*, 1961).

Two forms of the enzyme were prepared and partially purified from rat and rabbit skeletal muscle (Rosell-Perez and Larner, 1964a; Rosell-Perez *et al.*, 1962). They were differentiated from each other by the kinetics of the UDPG concentration dependence in the presence and absence of glucose-6-phosphate. In the case of the *D* (dependent) form of the enzyme, the activity without added glucose-6-phosphate was low or absent. In the presence of glucose-6-phosphate the *V* for UDPG was markedly increased. The K_m

* Papers I through V of this series were titled "Studies on UDPG-α-glucan Transglucosylase. This work was supported in part by a grant from the National Institute of Arthritis and Metabolic Diseases of the United States Public Health Service (A-2366) and by a research career award (K6-AM-985) from the National Institutes of Health.

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[1] Abbreviations used in this work: UDPG, uridine diphosphoglucose; Tris, tris(hydroxymethyl)aminomethane; transferase, UDPG-α-1,4-glucan transferase; TPN, nicotinamide adenine dinucleotide phosphate; DEAE, diethylaminoethyl.