GUANOSINE DIPHOSPHATE MANNOSE*

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Uridine diphosphate glucose (UDPG)[1] has been isolated from yeast and found to act as coenzyme in the transformation of galactose-1-phosphate to glucose-1-phosphate (1, 2). Similar compounds in which the glucose residue is replaced by galactose (3), acetylglucosamine (4), an amino sugar derivative plus amino acids (5), or glucuronic acid (6) have been described.

This paper describes the isolation of a related compound which was first detected by paper chromatography of UDPG preparations purified by anion exchange and which appears to be guanosine diphosphate mannose (GDPM).

Isolation of GDPM—The starting material was a nucleotide mixture obtained from bakers' yeast. The UDPG content has been found to increase by a short period of autolysis (2), and hence in the preparation of UDPAG (4) it was found convenient to omit this treatment in order to obtain a higher ratio UDPAG:UDPG. Better results for GDPM were obtained by including the autolysis step.

The yeast nucleotides were extracted with 50 per cent ethanol, followed by precipitation with mercuric acetate. In the preparation of UDPG (2) this precipitate was separated in two fractions, one soluble and another insoluble in 1 M ammonium acetate. The soluble fraction was found to contain most of the UDPG and UDPAG, while nearly all of the GDPM remained in the insoluble fraction.

In the further purification of GDPM the nucleotide mixture obtained by decomposition of the mercury salts with H_2S was fractionated with an anion exchange column. In some cases GDPM and UDPG were eluted from the column in a single peak, and the two substances had to be separated by paper chromatography. When the UDPG content of the extract

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[1] The following abbreviations will be used: UDPG for uridine diphosphate glucose, GDPM for guanosine diphosphate mannose, UDPAG for uridine diphosphate acetylglucosamine, AMP-5′ for adenosine-5′-phosphate, ADP for adenosinediphosphate, UMP-5′ for uridine-5′-phosphate, UDP for uridine diphosphate, GMP-5′ for guanosine-5′-phosphate, and GDP for guanosine diphosphate.

was not very high, as in the experiment shown in Fig. 1, a good separation was obtained. The GDPM-containing fractions were concentrated by adsorption and elution on charcoal. The product obtained was contaminated with a substance having the properties of guanosine-5′-phosphate. This compound was probably produced by decomposition of GDPM during the concentration process, since GMP-5′ emerged from the column much earlier

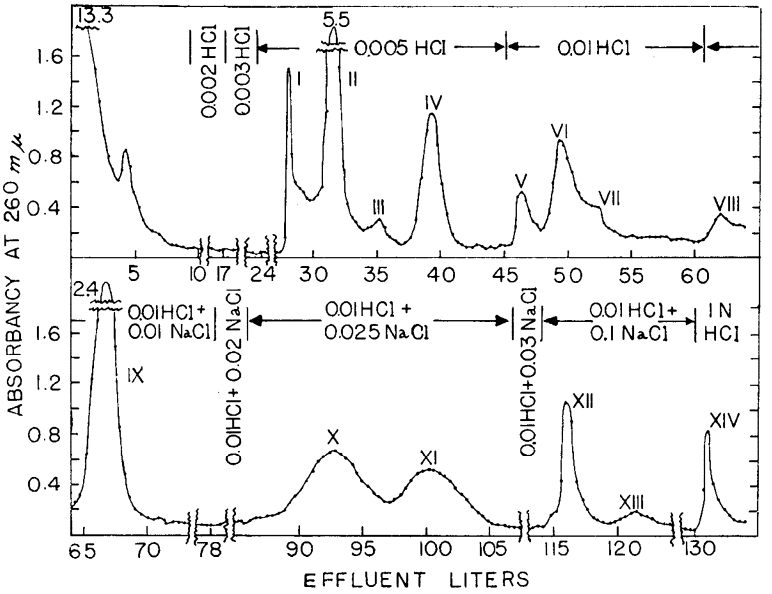


FIG. 1. Separation of yeast nucleotides by ion exchange (see Preparation 2 under "Methods"). The figures for HCl and NaCl represent the molarity. Probable identity of the substance in each peak: Peak II, AMP-5′; Peak IV, UMP-5′; Peak VI, GMP-5′; Peak VII, inosinic acid; Peak IX, ADP; Peak X, GDPM plus UDPAG; Peak XI, UDPG; Peak XII, UDP plus unidentified compound; Peaks I, III, V, VIII, XIII, and XIV, unidentified substances.

than GDPM (Fig. 1). The two substances were separated by ionophoresis on starch, after which GDPM was precipitated as the calcium salt.

Nucleotide Moiety—The ultraviolet absorption spectrum of the substance is presented in Fig. 2. The curve is nearly identical to that of guanosine and shows the same changes in acid and in alkaline solution.

Calculations based on phosphate estimations and on the guanosine content obtained from absorbancy values gave two phosphate groups per guanosine residue.

The hydrolysis curves of the phosphate groups are presented in Fig. 3. In 0.1 N acid at 100° 50 per cent of the phosphate was liberated in 120 min-

utes. There was a break in the curve for 1 N acid at 100° at 50 per cent hydrolysis, and the second part of the curve was parallel to that of guanosine-5′-phosphate. In the case of UDPG, the rate of hydrolysis of the

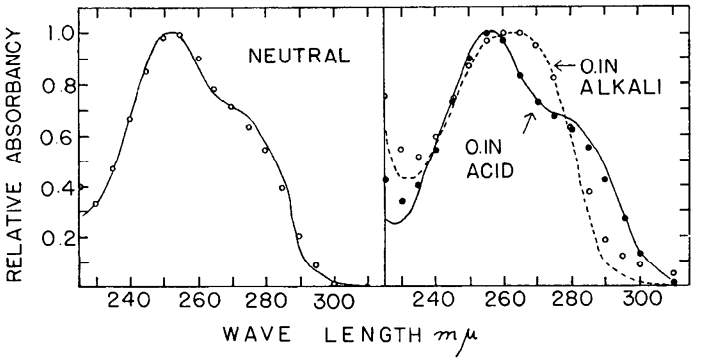


FIG. 2. Absorption spectra of GDPM and guanosine. The absorbancy value at the maximum was taken as equal to 1. Solid and broken lines, guanosine (from the data of Hotchkiss (7)); closed and open circles, GDPM.

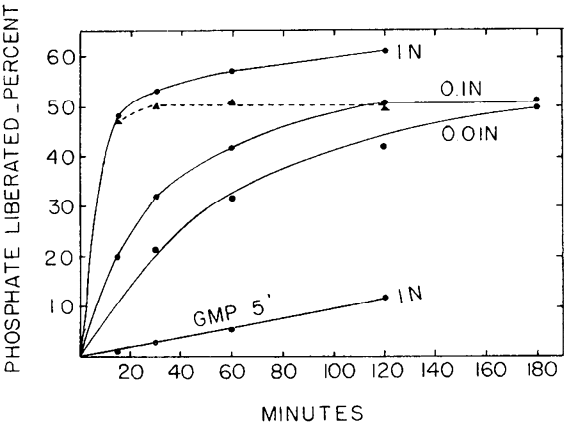


FIG. 3. Hydrolysis curves for phosphate of GDPM in acid at 100°. The percentages for GMP-5′ were divided by 2 in order to obtain a curve directly comparable with that of GDPM. The broken line was obtained by subtracting the values for GMP-5′ from those for GDPM in 1 N acid.

acid-labile phosphate group is similar to that of GDPM, but the second phosphate group is more stable. This agrees with the known difference in stability of the 5′-phosphates of uridine and guanosine (8).

Sugar Moiety—Mild acid hydrolysis of GDPM leads to the liberation of a reducing substance. By paper chromatography of this substance with two different solvents, followed by spraying with aniline phthalate (9) or

benzidine reagent (10), a single spot was obtained, of which the position on the paper and the color were the same as for authentic mannose. In order to confirm the identity of the sugar, an independent method, ionophoresis on borate-buffered paper (11), was used. The results are shown in Table I.

TABLE I

Paper Chromatography and Ionophoresis of Sugar from GDPM

Ionophoresis was carried out on a Whatman No. 1 sheet, 57 cm. long and 9.2 cm. wide. A potential of 500 volts was applied during 7 hours.

Substance	Chromatography		Ionophoresis
	*R*xylose with pyridine-ethyl acetate-water	*R*ribose with phenol-ammonia	*R*glucose with borate buffer
Glucose	0.83	0.61	
Galactose	0.71	0.70	0.88
Fructose	0.87	0.83	0.86
Mannose	0.91	0.71	0.64
Sugar from GDPM	0.90	0.71	0.66

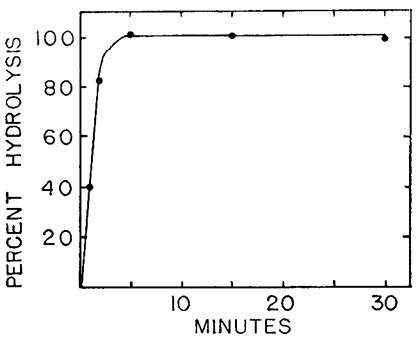


FIG. 4. Liberation of mannose from GDPM heated in 0.01 N acid at 100°. The value obtained after 15 minutes was taken as 100.

Mannose is very easily liberated from GDPM by 0.01 N acid, as can be observed in Fig. 4. As unhydrolyzed GDPM is non-reducing, it may be concluded that mannose is linked to the rest of the molecule through carbon atom 1.

Acid Hydrolysis of GDPM—When GDPM was heated during 15 minutes at 100° at its own acidity (concentration of the solution, 2 μM per ml.; pH about 2.7), all the mannose and very little phosphate (about 10 per cent) were liberated. Thus, guanosine diphosphate was expected to be present in the hydrolysate. Accordingly, chromatograms with two different sol-

vents showed a slow moving, ultraviolet-absorbing spot, which gave a positive reaction with the Hanes and Isherwood spray reagent for phosphate (12) (see Table II). The reaction with the benzidine color reagent for sugars was negative, while it was slightly positive with GDPM.

Hydrolysis of GDPM under the same conditions as above, but during 180 minutes, resulted in the liberation of about half the total phosphate

TABLE II

Paper Chromatography of GDP and GMP from GDPM

Substance	$R_{adenosine}$ values of ultraviolet-absorbing spots	
	Ethanol-ammonium acetate, pH 7.5	Ethanol-ammonium acetate, pH 3.8
GDPM	0.31	0.29
" heated 15 min., pH 2.7, 100°	0.12	0.24
" " 180 min., pH 2.7, 100°	0.18	0.43
Synthetic GMP-5′	0.19	0.43
Yeast guanylic acid	0.23	0.60

TABLE III

Action of 5′-Nucleotidase on Guanosine Monophosphate from GDPM

The technique was as described by Cabib *et al.* (4). The amount of substrate was 0.24 μM per tube. The $R_{uridine}$ of authentic guanosine was 0.79.

Substrate added	Time of incubation	Inorganic phosphate	$R_{uridine}$ of ultraviolet-absorbing substances
	min.	*μM*	
GMP from GDPM	0	0.03	0.22
" " "	30	0.17	0.74
Synthetic GMP-5′	0	0.02	0.21
" "	30	0.19	0.73
Yeast guanylic acid	0	0.03	0.27
" " "	30	0.03	0.27

and about 10 per cent of the guanine. The latter precipitated upon neutralization and was identified by its absorption spectrum. The supernatant liquid was submitted to paper chromatography with two different solvents. An ultraviolet-absorbing, phosphate-containing spot was observed. Its $R_{adenosine}$ value was the same as that of synthetic guanosine-5′-phosphate and clearly different from that of yeast guanylic acid (see Table II).

The position of the phosphate group in the guanylic acid was further confirmed by treatment with a specific 5′-nucleotidase from snake venom (13). As can be seen in Table III, both the guanylic acid obtained from

GDPM and synthetic GMP-5′ were hydrolyzed by the enzyme, yielding inorganic phosphate and a substance with an R_{uridine} value similar to that of guanosine, while yeast guanylic acid was not attacked. The small difference between the R_{uridine} values of authentic guanosine and of the guanosine obtained from the guanylic acids is to be ascribed to the presence of salts in the enzymatic hydrolysates.

Chromatography of the products of hydrolysis of GDPM in 1 N acid during 30 minutes at 100° revealed the presence of a substance which behaved like guanine. The R_F values with an isopropanol-hydrochloric acid solvent (14) were 0.26 for hydrolyzed GDPM, 0.25 for guanine, and 0.39 for adenine.

TABLE IV

Analytical Data for Sample of Calcium Salt of GDPM

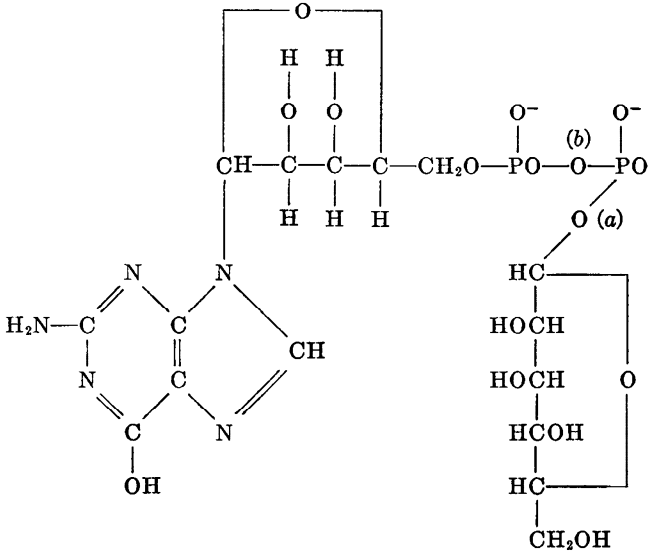
The substance was dried over phosphorus pentoxide at 56° during 4 hours. In order to minimize the error due to non-specific ultraviolet-absorbing substances, the guanosine concentration was calculated from the absorbancy at two wave-lengths in neutral solution as follows. Guanosine concentration (micromoles per ml.) = $((A_{250} - A_\lambda)/(a_{m,250} - a_{m,\lambda})) \times 10^3$, where A_{250} represents the absorbancy of the sample at 250 mμ; $a_{m,250}$ the molar absorbancy at 250. The corresponding values at another wave-length are represented as A_λ and $a_{m,\lambda}$. With the data of Hotchkiss (7) the same results were obtained by setting λ equal to 230 or 280 mμ.

Component	Found	Theoretical for Ca salt	Moles of component; total phosphate taken as 2.00
	μM per mg.	*μM per mg.*	
Guanosine	1.28	1.55	1.08
Total phosphate	2.36	3.1	2.00
Labile phosphate, 120 min. in 0.1 N acid, 100°	1.23	1.55	1.04
Mannose	1.04	1.55	0.88

Structure of GDPM—The analytical data for the calcium salt of GDPM appear in Table IV. The results correspond to a preparation of about 75 per cent purity of a compound containing one guanosine, two phosphate groups (one of them being acid-labile), and one mannose residue.

The fact that the intact compound is non-reducing and that a guanosine diphosphate and GMP-5′ are liberated by acid hydrolysis suggests a structure similar to that of UDPG and UDPAG, as represented in the accompanying formula. Such a compound, after being passed through a cation exchange resin in the acid form, should show on electrometric titration two acid groups, one dissociating as a primary phosphate and the other as the ammonium cation of guanosine (15). Hydrolysis at the points marked *a* and *b* would yield one secondary phosphoric acid group in each case. Be-

sides, hydrolysis at *a* and *b* would liberate mannose and inorganic phosphate respectively. Therefore, the electrometric data were compared with



the values predicted from total phosphate, inorganic phosphate, and free mannose estimations in samples of the substance, before and after hydrolysis. The results, summarized in Table V, while not so clear cut as in the

TABLE V

Electrometric Titration of GDPM

The technique was as described previously (2).

Sample	Time of heating at 100°, pH about 2.7	μeq. of base			
		Calculated from analytical data		Observed on electrometric titration	
		Primary	Secondary	Primary*	Secondary†
	min.				
A	0	5.37	0	5.84	0‡
B	15	6.0	3.51	6.9	3.2
C	180	6.2	5.94	6.4	5.6

* Corresponds to the dissociation of a primary phosphate group and the ammonium group of guanosine. Titrated to pH 4.5.

† Titrated from pH 4.5 to 8.2.

‡ A small amount of alkali (1.3 μeq.) was used to shift the pH from 4.5 to 8.2, but the buffering action was at a minimum around the pK zone of secondary phosphate; that is, from pH 6 to 7.5. Therefore, it was assumed that this amount of alkali was used to titrate impurities, and it was subtracted from all the titrations.

case of UDPG and UDPAG (2, 4), are, however, substantially consistent with the proposed structure.

DISCUSSION

Buchanan *et al.* (16) have reported the presence of several nucleotide-bound hexoses in green plants and algae. The evidence indicated that glucose and galactose were present as UDP-glucose and UDP-galactose. A combined form of mannose, presumably GDPM, was also found in the same fraction.

The function of one of these nucleoside-pyrophosphate-sugar compounds as donor of the sugar moiety has been proved by Dutton and Storey (6). They found that UDP-glucuronic acid acts as a glucuronyl donor in the synthesis of glucuronides by liver enzymes. A somewhat similar rôle for UDPG in the synthesis of saccharose phosphate has been postulated by Buchanan *et al.* (16, 17). By analogy it may be assumed that GDPM and UDPAG function as donors of mannose and acetylglucosamine residues, respectively, and that they are involved in the synthesis of mannan and chitin, which are present in the yeast wall (18).

Methods

Methods were in general the same as those employed in previous papers (2, 4).

Paper chromatography of sugars was carried out with ethyl acetate-pyridine-water (3, 19), or with phenol-ammonia (20). The chromatograms were sprayed with aniline phthalate (9) or benzidine-trichloroacetic acid (10).

For the chromatography of nucleotides and nucleosides, the ethanol-ammonium acetate mixtures already described (21) were used. In order to counteract the "tailing" shown by some of the compounds, a small amount of sodium Versenate (sodium salt of ethylenediaminetetraacetic acid) was added to the solvents (*cf.* Walker and Warren (22)). The concentration of chelating agent was 10^{-2} M for the solvent of pH 7.5 and 10^{-3} M for that of pH 3.8.

After examination for ultraviolet-absorbing spots with a Mineralight lamp, the chromatograms were often sprayed successively with benzidine-trichloroacetic acid and the Hanes and Isherwood molybdate reagent (12) to ascertain the position of sugar- and phosphate-containing substances.

Ionophoresis on paper of sugars and nucleotides was performed with an apparatus similar to that devised by Kunkel and Tiselius (23). A 0.05 M borax solution (pH about 9.2) was employed for sugars (11), and a 0.05 M ammonium acetate buffer of pH 3.8 for nucleotides. In chromatographic as well as in ionophoretic experiments, the position of the substances on

the paper was usually referred to the position of an appropriate standard (glucose, xylose, or ribose for sugars, and adenosine or uridine for nucleotides); the results are given in the form

$$R_{\text{standard}} = \frac{\text{distance traveled by unknown}}{\text{distance traveled by standard substance}}$$

Preparation of GDPM

Preparation 1—In this case a nucleotide mixture, obtained as for the preparation of UDPAG but from toluene-treated yeast (950 ml. containing about 6000 μM of nucleotides calculated as uridine from the absorbancy at 260 mμ), was submitted to chromatography on the same Dowex 1 column previously employed for UDPAG (4). Considerable overlapping occurred between the UDPAG and UDPG peaks, eluted with 0.03 N sodium chloride in 0.01 N hydrochloric acid, owing probably to the relatively small amount of UDPAG present (*cf.* Cabib *et al.* (4)). The fractions corresponding to the UDPG peak were pooled and concentrated as previously described (4) and then chromatographed on blotting paper sheets (24) with ethanol-ammonium acetate of pH 3.8. The $R_{\text{adenosine}}$ values with this solvent were usually about 0.30 for GDPM, 0.45 for UDPG, and 0.60 for UDPAG. The blotting paper was prewashed with the same solvent and then with ethanol and distilled water successively. After chromatography, the ultraviolet-absorbing zone corresponding to GDPM was cut off, washed with ethanol to remove most of the ammonium acetate, and eluted with distilled water. The yield was about 25 μM.

Preparation 2—10 kilos of bakers' yeast were submitted to the same initial steps as for the preparation of UDPG (2). The precipitate of the mercury salts of nucleotides (Step 2) was extracted with 1200 ml. of 1 M ammonium acetate. The insoluble fraction was blended with 1000 ml. of water and decomposed with hydrogen sulfide in the cold. After filtering, the resulting solution was aerated and neutralized. It was found by acid hydrolysis of small samples, followed by paper chromatography, that this fraction contained most of the acid-labile mannose. The extract (960 ml., containing about 8000 μM, calculated as above) was run on the Dowex 1 resin column as for Preparation 1 (see Fig. 1). The substances corresponding to the different peaks were tentatively identified by their ultraviolet spectra, phosphate content, and chromatographic behavior, often by comparison with the nucleotides isolated previously (4). From Peak X about 340 μM of GDPM (calculated from the absorbancy at 260 mμ), contaminated with small amounts of UDPAG, were obtained. The substances were concentrated by adsorption and elution on charcoal and precipitated by stepwise addition of ethanol in the presence of calcium chloride (4). The calcium salt of GDPM, which is less soluble, precipitates in the first

fractions practically free from UDPAG. The preparation thus obtained was contaminated with a substance which migrated like GMP-5′ on paper chromatography or ionophoresis. The latter procedure was selected for the preparative separation. Several fractions of the calcium salt of GDPM, totaling 42 mg., were pooled and dissolved in water, after which the calcium was removed with ammonium oxalate. The supernatant liquid was submitted to ionophoresis on starch as described by Kunkel and Slater (25). A starch slab, 45 cm. long × 5 cm. wide × 0.5 cm. thick, moistened with 0.05 M ammonium acetate buffer of pH 3.8, was employed. The sample (0.7 ml.) was spotted at 4.5 cm. from the cathodic end along a transversal depression, which was afterwards replenished with barely moist starch. Ionophoresis was carried out during 6 hours under a potential of 750 volts. The position of the substances was ascertained with the Mineralight lamp. The GDPM band, which had migrated faster toward the anode, was cut off and extracted with two 75 ml. portions of water on a Büchner funnel. The water extracts were pooled, evaporated to dryness *in vacuo*, and left during 3 days at 35° in a vacuum desiccator containing sodium hydroxide and sulfuric acid to remove as much ammonium acetate as possible. The residue was redissolved in water, and GDPM precipitated as the calcium salt with aqueous ethanol. Yield, 21 mg.

Preparations 1 and 2 were chromatographically identical, but Preparation 2 was purer, as judged from the analytical data, and was used for all the determinations, except for the acid hydrolysis curve of phosphate and the identification of guanine.

Degradation Products of GDPM

Identification of Mannose—For the quantitative tests, GDPM was hydrolyzed with 0.05 N sulfuric acid during 15 minutes at 100°, the mixture was neutralized with 0.06 N barium hydroxide, and the nucleotides were precipitated by adding equal volumes of 5 per cent zinc sulfate and 0.3 N barium hydroxide. After centrifuging, mannose was determined in an aliquot of the supernatant liquid with an adaptation of the Schales and Schales ferricyanide method (26).

To obtain a solution of the sugar suitable for chromatography or ionophoresis, an aliquot of Sample B, previously used for the electrometric titration (see Table V), was passed successively through cation exchange and anion exchange resin columns (Dowex 50 and Amberlite IR-4B respectively). The solution was then evaporated *in vacuo*, and samples were taken for chromatography or ionophoresis on paper (Table I).

Guanosine Diphosphate—Aliquots of Sample B from the electrometric titration (Table V) were evaporated *in vacuo* and used for chromatography (Table II).

Guanosine Monophosphate—An aliquot from Sample C, used for the electrometric titration (Table V), was centrifuged to separate the guanine which had precipitated, and the supernatant liquid was divided in two portions. One was evaporated *in vacuo* and used for paper chromatography (Table II), while the other was brought to pH 9 with 0.3 N barium hydroxide to precipitate inorganic phosphate. After centrifuging, the supernatant fluid was neutralized with sulfuric acid and centrifuged again. The resulting solution was evaporated *in vacuo* and used for the incubation with 5′-nucleotidase (see Table III).

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SUMMARY

The isolation of a mannose-containing nucleotide from yeast is reported. Mannose was identified by chromatography and ionophoresis on paper. The nucleotide was found to give an ultraviolet absorption spectrum nearly identical to that of guanosine and to contain two phosphate groups. Mannose could be liberated by mild acid hydrolysis. Further hydrolysis led to the removal of one phosphate group, leaving a substance which behaved like guanosine-5′-phosphate on paper chromatography and when treated with 5′-nucleotidase. After treatment with stronger acid, a substance with the properties of guanine was detected. The properties of the compound, including the titration curves, are consistent with those of a structure in which the terminal phosphate of guanosine-5′-pyrophosphate is joined to a mannosyl residue.

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