A Synthesis of Uridinediphospho-*N*-acetylmuramic Acid and Its Use as an Acceptor of L-[^{14}C]Alanine*

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ABSTRACT: On the basis of model studies, methyl triacetyl-*N*-acetylmuramate, largely α form, was treated with H_3PO_4 under conditions optimal for production of hexosamine-bound phosphate (18% yield). Both anomers were formed but ion exchange failed to separate them. Reaction with uridine 5′-phosphomorpholidate gave uridinediphospho-*N*-acetylmuramic acid, isolated in 13% yield; the salt-free product was homogeneous on paper and thin-layer chromatography and exhibited the expected stoichiometry, but in all likelihood is a mixture of the α and β anomers. One-half of the synthetic material serves as an acceptor of [^{14}C]alanine when it is incubated with a cell-free preparation obtained from cultures of group A hemolytic streptococci. The product, uridinediphospho-*N*-acetylmuramyl-L-alanine, was purified and characterized.

The title compound is a precursor of bacterial peptidoglycan; it has been isolated from penicillin-inhibited cultures of *Staphylococcus aureus* as one of the so-called "Park nucleotides" (Park and Johnson, 1949) and from cultures of group A *Streptococcus pyogenes* (Cifonelli and Dorfman, 1957). Our interest in the biosynthesis of peptidoglycan led us to undertake a chemical synthesis of the nucleotide. A preliminary report of the work has been given (Heymann *et al.*, 1966).

To obtain the requisite *N*-acetylmuramic acid 1-phosphate (MurNAc-1-P),[1] MacDonald's procedure of fusing a fully acetylated sugar with anhydrous phosphoric acid (MacDonald, 1962) appeared attractive. The applicability of the method to amino sugars was explored by subjecting α- and β-tetra-*O*-acetylglucosamine to the action of anhydrous phosphoric acid. The reaction mixtures were processed by saponification with lithium hydroxide and fractionation on Dowex 1 (Cl^-) resin with gradients of ammonium chloride or lithium chloride. The yield of organically bound phosphate in the effluents was 56%, based on pentaacetyl-D-glucosamine; the ratio of anomers was approximately $\alpha:\beta = 40:60$, regardless of the anomerism of the starting pentaacetate.

Separation of the anomeric phosphates by means of salt gradient elution was moderately satisfactory. Ammonium chloride gave better separation than lithium chloride, but is more difficult to remove from the product by alcohol precipitation. After our work was completed, Olavesen and Davidson (1965) and O'Brien (1964) reported their experiences with phosphoric acid fusion of pentaacetyl-D-glucosamine. O'Brien separated the anomeric phosphates by means of a hydrochloric acid gradient in the cold, with results superior to ours. The acid gradient is preferable to the salt gradient, unless excessive lability of one of the anomers demands the use of a neutral solvent.

Methyl tri-*O*-acetyl-*N*-acetylmuramate, 2-acetamido-1,4,6-tri-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-D-glucopyranose, was obtained from methyl *N*-acetylmuramate, 2-acetamido-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-D-glucopyranose (Carroll, 1963; Flowers and Jeanloz, 1963; Osawa and Jeanloz, 1965) by means of pyridine and acetic anhydride (Carroll, 1963). Separation of anomers was not undertaken since in the case of pentaacetylglucosamine (*vide supra*) the end product was independent of the anomer initially employed.

The effects of time, temperature, solvent, and of added phosphorus pentoxide on the fusion reaction were studied. The formation of organic phosphate was used as an indicator, and the two last-named variables were seen to have no, or a negative effect. Direct fusion for 3 hr at 50° or for 45 min at 90° was the most favorable condition, but even so the yield of organic phosphate was only 18%.

Elution patterns of the saponified phosphate esters from Dowex 1 (Cl^-) indicated the presence of two anomers but none of the conditions tried afforded an effective separation. Moreover, there is some reason to believe that anomerization takes place very readily (see Experimental Section). Elution with dilute acid and conversion into the lithium and then the potassium salt yielded a product of 97% purity on the basis of phosphate and *N*-acetylmuramic acid. Of the total ester phosphate, about 50% was released under very mild conditions, indicating an anomeric composition of approximately 1:1. Whereas in the case of the acetylglucosamine 1-phosphates the α anomer (high positive rotation, acid-resistant phosphate) emerges from the Dowex 1 columns before the β isomer (low or zero rotation, acid-labile phosphate), the reverse holds for

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[1] Abbreviations used: MurNAc, *N*-acetylmuramic acid; MurNAc-1-P, *N*-acetylmuramic acid 1-phosphate; UDPMurNAc, uridinediphospho-*N*-acetylmuramic acid; UDPMurNAc-L-Ala, uridinediphospho-*N*-acetylmuramyl-L-alanine.

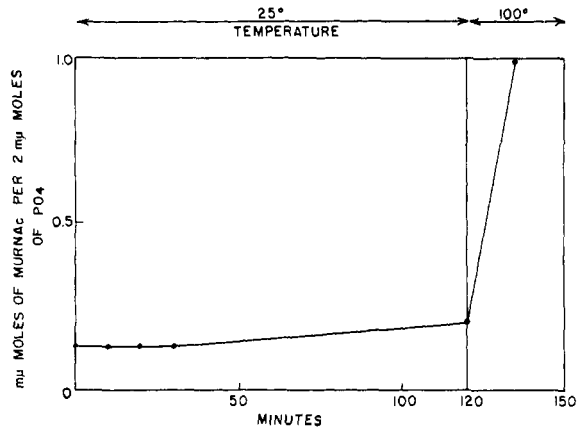

FIGURE 1: Liberation of *N*-acetylmuramic acid from UDP-MurNAc. Aliquots of 500 μl contained 52 μg of Li_3UDP-MurNaC·$3H_2O$ in 0.01 N HCl. At the times specified, 500 μl of sodium tetraborate 0.1 M in B was added, except in the case of the zero-time sample for which the order of addition of acid and borate was reversed. The Morgan–Elson reaction was performed by heating at 100° for 3 min, addition of 2.5 ml of dimethylaminobenzaldehyde reagent and 20-min incubation at 37°.

the anomers of acetylmuramic acid 1-phosphate. Early fractions from the broad elution peaks generally exhibited a lower rotation than later tubes. Evidently, those features permitting differential elution in the case of the glucosamine anomers are being counteracted by an effect of the carboxyl side chains. The possibility of effecting a separation at the methyl ester stage has not yet been evaluated. The purified, dried product gave good analytical values for phosphorus, *N*-acetylmuramic acid, H, and N, although the C analysis did not conform to standards customarily applied to organic preparations.

The unseparated anomeric mixture was used for conversion into the nucleotide sugar, uridinediphospho-*N*-acetylmuramic acid (UDPMurNAc), by means of uridine 5′-phosphoromorpholidate according to well-known procedures (Roseman *et al.*, 1961).

The total yield of identifiable UDPMurNAc amounted to 13.4% of the theoretical quantity;[2] the major portion of the product was obtained in the form of analytically satisfactory trilithium salt. The product was purified by elution from Dowex 1 (Cl^-), charcoal adsorption, and alcohol precipitation. The substance exhibited the expected stoichiometry and the analytical data suggested the presence of a trihydrate (see Table I). Thin-layer and paper chromatograms as well as paper electropherograms exhibited only one spot of ultraviolet-absorbing, phosphorus-containing material. The substance was stable to cold acid (see Figure 1).

Our preparation of UDPMurNAc is capable of accepting [^{14}C]alanine in the presence of a suitable fraction derived from *S. pyogenes*, analogous to the experiments with *S. aureus* by Ito and Strominger (1960, 1962). Optimal conditions for ^{14}C incorporation were established for the purpose of using the alanine-adding reaction as a sensitive analytical tool for the detection of biosynthetic UDPMurNAc from UDP-GlcNAc in cell homogenates of *S. pyogenes* in pursuit of our interest in muramic acid biosynthesis (Barkulis *et al.*, 1967). We were able to isolate a small amount of the enzymatic product free from residual UDP-MurNAc and showed that it contained [^{14}C]alanine and phosphorus in a ratio close to 1:2.

TABLE I: Analyses of Li_3UDPMurNAc·$3H_2O$.

	Calcd (%)[a]	Found (%)	Ratio
Phosphorus	8.2	8.3	1.99
N-Acetylmuramic acid	39.0	38.7	0.99
Uridylic acid (based on E_{262} 10,000)	43.2	43.5	1.00

[a] For $C_{20}H_{28}N_3O_{19}P_2Li_3 \cdot 3H_2O$ (751.3).

The synthetic UDPMurNAc was suitable for the purpose just outlined. However, in the context of the present report, the question of its anomeric purity must be discussed. The coupling reaction between uridine phosphoromorpholidate and the anomeric mixture of amino sugar 1-phosphates might have proceeded in only one sense, because of the apparent lability of the β anomer or because of a preferential rate of reaction for the α anomer; on the other hand, it might have produced an anomeric mixture.

Two examples in the literature make it plausible that hexose β-1-phosphates react normally, at least in the phosphoramidate procedure: UDP-β-glucose (Ueda, 1960) and ADP-β-glucose (Recondo and Leloir, 1961). Thus one cannot reason that β anomers react with difficulty.

The product, UDPMurNAc, resists 0.01 M HCl at room temperature (see Figure 1), conditions under which the labile anomers of GlcNAc-1-P and MurNAc-1-P liberate *N*-acetylhexosamine. However, the stability relations existing among the sugar 1-phosphate anomers do not necessarily pertain to the corresponding uridinediphospho sugars. The papers by Ueda and by Recondo and Leloir cited above do not give any data on the relative acid stability of the β anomers described.

In Table II, we are comparing the changes in molecular rotation ($M_D = [\alpha]_D \times$ mol wt $\times 10^{-2}$) exhibited by three hexoses on conversion from the α anomer into the corresponding UDP derivative with the change in the M_D given by MurNAc when converted into the preparation of UDPMurNAc described here.

The literature value cited for the specific rotation of α-MurNAc is the one recorded immediately after dissolution. The figure probably, but not certainly, pertains to the α anomer. Even if the value for the

[2] The figure of 46% appearing in our preliminary report (Heymann *et al.*, 1966) is in error. Although more UDPMurNAc was formed than the 13.4% isolated, the amount lost during the purification is unlikely to exceed 10–15% of the theroetical yield.

TABLE II: Change of Molecular Rotation Attending Conversion of Hexose into UPD-hexose.

Hexose	$[\alpha]_D^{25}$ of Hexose (deg)	M_R of Hexose (deg)	$[\alpha]_D^{25}$ of UDP-hexose (deg)	M_R of UDP-hexose (deg)	ΔM_R (deg)
α-Glucose	+113	+204	+43.6[c]	+300	+96
α-Galactose	+151	+272	+42.9[d]	+248	−24
α-GlcNAc	+64[a]	+142	+54.6[d]	+338	+196
α-MurNAc	+60[b]	+176	+20 ± 2[e]	+139	−37

[a] White (1938). [b] Osawa and Jeanloz(1965). [c] Value given for lithium salt hexahydrate by Moffatt and Khorana (1958). [d] Value calculated for anhydrous lithium salt; Roseman *et al.* (1961). [e] Value determined for the anhydrous trilithium salt described in the present report. The measurement was performed in a Perkin-Elmer 141 polarimeter; *c* 0.1, H_2O.

TABLE III: Incorporation of [^{14}C]Alanine into Uridinediphospho-*N*-acetylmuramic Acid.[a]

mμmoles in Reaction Mixture		Moles of Ala/Mole of UDPMurNAc	Radioactivity[c] in Nucleotide (% incorp)	% of UDPMurNAc Utilized
UDPMurNAc	[^{14}C]Alanine[b]			
22.5	11.2	0.5	29.9	14.9
11	11.2	1	22.5	22.4
5.6	11.2	2	14.5	29
1.12	11.2	10	4.55	45.6
Nil	11.2		Nil	Nil

[a] Each incubation tube contained 160 μg of enzyme protein. See text for the other constituents of the reaction mixture. [b] The radioactivity of this quantity of alanine was 80,000 cpm. [c] Average of two determinations.

pure α anomer should, at a later date, be found to be higher, the result would only be a greater drop in molecular rotation than the one given in Table II for the change attending the conversion of MurNAc into the preparation of UDPMurNAc described here. The validity of comparisons of molecular rotation is debatable, but one should note that the change has a large positive value for the two members of the D-glucose family but is negative for the sample of UDP-MurNAc. The tentative conclusion to be drawn from Table II is that the sample of UDPMurNAc represents a mixture of anomers.

The biological activity of the UDPMurNAc preparation as an acceptor of [^{14}C]Ala is illustrated in Table III, where it can be seen that on increasing the molar proportion of alanine 45–50% of the UDPMurNAc could be made to accept the amino acid. In other experiments the ratio of alanine to nucleotide was varied from equivalence to 100-fold excess, but in no case did the nucleotide-bound radioactivity exceed 45–50% of the amount expected on the basis of UDP-MurNAc present. It is not known how well natural UDPMurNAc would be utilized when present in limiting amounts, and equilibrium conditions might be unfavorable to quantitative conversion even in the presence of excess amino acid.

However, the sum total of the evidence considered here makes it probable that our preparation is a mixture of approximately equal parts of the α and β anomers of UDPMurNAc, and that the lability to acid so characteristic of the β-1-phosphates is not carried over to the uridinediphospho derivative. Possibly anomer separation can be accomplished at the nucleotide sugar stage by ion-exchange chromatography, but at present we have no evidence on this point.

Experimental Section

Methods and Materials

The Morgan–Elson reaction was performed according to the modification of Reissig *et al.* (1955). Total phosphate was determined according to Fiske and Subbarow (1925) or Martin and Doty (1949), after ashing with perchloric or sulfuric acid. "Labile" and "total" 1-phosphate esters were measured by determining the amount of orthophosphate liberated on exposure to 0.5 N mineral acid at room temperature for 30 min, and at 100° for 10 min. Inorganic phosphate was removed, when necessary, by centrifugation as $MgNH_4PO_4$. The Elson–Morgan test for free hexosamine was carried out as modified by Boas (1953).

The majority of the paper chromatograms were developed with ethanol–ammonium acetate (1 M, pH 7.5, 7:3) referred to as solvent A in the text. Other solvents employed are specified at the appropriate point.

Salts of N-Acetylmuramic Acid 1-Phosphate, 2-Acetamido-3-O-(D-1-carboxyethyl)-2-deoxyglucopyranose 1-Phosphate. 1. PHOSPHORYLATION. The following describes a typical run. Methyl tri-*O*-acetyl-*N*-acetylmuramate, 2-acetamido-2-deoxy-3-*O*-[D-1-(methyl carboxylate)ethyl]-1,4,6-tri-*O*-acetylglucopyranose (3.37 g, 7.78 mmoles), unresolved anomeric mixture, and crystalline phosphoric acid (3.12 g, 196 mequiv) were dried *in vacuo* for 48 hr over $Mg(ClO_4)_2$. The phosphoric acid was melted in a 100-ml flask at 50° in an oil bath, the finely powdered sugar acetate was added, and the mixture was heated in an oil bath maintained between 52 and 57°, with continuous evacuation. After 3 hr an Ascarite tube in the evacuation train showed the weight gain expected from liberation of 1 equiv of acetic acid. The dark brown mixture was taken up in 30 ml of tetrahydrofuran and treated under ice cooling with 252 ml of 0.925 N LiOH (10% excess over the amount needed to neutralize the phosphoric acid and to saponify the organic ester groups). The mixture stood overnight at room temperature. After filtration from lithium phosphate, 308 ml of yellow solution resulted; it was adjusted to pH 8 with 0.1 N HCl. The solution contained 1.39 mmoles of organic phosphate of which 43% was labile to 0.5 N acid at room temperature, presumably the β isomer. The crude organic phosphates correspond to a yield of 18% in the phosphorylation. Addition of solvents, of P_2O_5, or variation in temperature and duration of reaction did not improve the conversion and in many instances decreased the yield.

2. LITHIUM SALT. One-half of the above filtrate was concentrated to a thick syrup under reduced pressure at 40–45°. By addition of ethanol a sticky solid was caused to separate. The supernatant was decanted and the sediment was triturated with fresh alcohol. The resulting solid weighed 107 mg. By concentration and precipitation of the alcoholic supernatant, two more crops totaling 160 mg of tan solid were obtained. The salt was very hygroscopic. Analyses for MurNAc and phosphate both indicated that the solid contained 70% of anhydrous trilithium salt. Weight loss on drying at 100° *in vacuo* ranged from 5 to 11%. Chromatography in solvent A revealed one phosphate spot (R_F 0.33). The lithium salt was also obtained by passage of a solution of the ammonium salt over Dowex 50 W (Li form).

3. BARIUM SALT. To 50 mg of the lithium salt in 0.5 ml of H_2O was added the solution of 40.1 mg of barium acetate in 0.5 ml of H_2O. On addition of 7 ml of alcohol, a precipitate formed, which was sedimented, washed with alcohol, and dried at 100° *in vacuo*. The yield was 56 mg. The Fiske–Subbarow phosphate analysis (after removal of $BaSO_4$) gave variably high results, depending on the length of hydrolysis of the phosphate ester. However, on ashing the sample or with the use of the Martin–Doty method the phosphate content was found to be 4.5–4.6%. The content of MurNAc was 37.5%. The values expected for pure anhydrous $(MurNAc\text{-}1\text{-}P)_2Ba_3$ are 5.4 and 50.5%, respectively. The salt was nonhygroscopic but its high phosphate content made it unsuitable as a means of purification.

4. POTASSIUM SALT. Lithium *N*-acetylmuramic phosphate (about 0.15 mmole) was passed through a 10-ml column of Dowex 50-W (K form), the column was washed with water, and the potassium salt was recovered by precipitation with alcohol. It was reprecipitated twice with alcohol and once with acetone–alcohol. The recovery was 80–90%. *Anal.* Calcd for $C_{11}H_{17}K_3NO_{11}P \cdot 3H_2O$ (541.6): C, 24.4; H, 4.28; N, 2.58; P, 5.72; MurNAc, 54.1. Found: C, 26.15; H, 4.53; N, 2.51; P, 5.69; MurNAc, 53.

5. AMMONIUM SALT. Elution of the phosphates from Dowex 1 (Cl^-) columns with NH_4Cl gave a solution of the ammonium salt of *N*-acetylmuramic acid 1-phosphate, which exhibited equivalent contents of phosphate and *N*-acetylhexosamine. However, no adequate means was found to free the desired salt from accompanying ammonium chloride, except conversion into the lithium salt as described in 2.

6. ATTEMPTED SEPARATION OF ANOMERS. The solution of crude lithium salts obtained from the phosphorylation of 1.86 g of methyl tri-*O*-acetyl-*N*-acetylmuramate as described in 1 and containing 0.76 mmole of ester phosphate was adjusted to pH 8 and applied to a 300-ml bed of Dowex 1-X8 Cl^-. Elution with 0.2 M ammonium chloride removed the Li^+ ions in the first 120 ml of effluent. At effluent volume 860–1110 ml, collected in 27 10-ml fractions, a total of 0.63 mmole of ester phosphate emerged as a broad peak with a slight saddle near the summit. Fractions 1–8, 9–19, and 20–27 were pooled and concentrated. Material eluted early- and late-exhibited optical rotations, α_D +0.029 and +0.49°, respectively (1-dm tube, normalized to 460 μg of P/ml). Almost all of the phosphate in the late fraction was stable to 0.5 N acid at room temperature, whereas the early fraction contained about 50% of labile phosphate. When the early and the middle cut (fractions 1–19) were reeluted from the Dowex 1 column with a linear gradient of 0.1–0.22 N NH_4Cl, phosphate emerged at 1750–2100-ml effluent volume with a chloride range from 0.17 to 0.2 M. The elution diagram showed improved but still incomplete separation. Recovery of the sugar phosphate by fractional precipitation with alcohol from aqueous solution was unsatisfactory because ammonium chloride was tenaciously retained. Moreover, a fraction that had appeared to contain only the labile (β) anomer showed, at the end of the work-up, 70% of its phosphate in acid-stable form, a finding that suggests facile anomerization. An experiment to destroy the acid-labile isomer preferentially by mild hydrolysis offered little promise and this approach was abandoned.

Elution with dilute HCl was not satisfactory, either, in separating the anomers. A solution of 0.22 mmole of lithium *N*-acetylmuramic acid 1-phosphate was added to 250 ml of Dowex 1 (Cl^-) in a 4.8 × 60 cm column kept at 4°. The column was eluted with 0.05 N HCl at 4°, and fractions of 240 ml were collected, each fraction being received in an erlenmeyer flask provided with 12 ml of 1 N NH_4OH. The bulk of the phosphorus emerged in fractions 2–4, and showed the following partition on differential analysis.

For fractions 2, 3, and 4 the ratio $P_{lab}:P_{stab}$ was 1:1.7, 1:2.3, and 1:4.75. Again, only incomplete separation was indicated.

Uridinediphospho-N-acetylmuramic Acid (UDPMurNAc). Uridine 5′-phosphoromorpholidate (75 μmoles) (Calbiochem, 64.5% material) and potassium *N*-acetylmuramic acid 1-phosphate (140 μmoles), converted into the tri-*n*-octylamine salt, were condensed in 5 ml of pyridine for 5 days at room temperature, according to the procedure of Roseman *et al.* (1961). After removal of the pyridine *in vacuo* and decomposition of the reaction mixture with ether and sodium acetate solution, the aqueous phase was passed over a 1 × 12 cm column of Dowex 1-X8 Cl^-. The column was washed with water and eluted with a linear gradient produced from a mixing vessel holding 1 l. of 0.1 M LiCl in 0.01 M LiOAc buffer (pH 5.5) and a reservoir holding 1 l. of 0.2 M LiCl buffered in the same way. Fractions of 5 ml were collected and monitored by measuring the absorption at 262 mμ in 100 aliquots diluted 50-fold with 0.1 N HCl. Positive tubes were found in the water wash, in tubes 1–20 after the beginning of the elution, and in tubes 118–147, in which the LiCl concentration ranged from 0.133 to 0.138. Tubes 118–124 (fraction I), 125–130 (fraction II), and 131–147 (fraction III) were pooled on the basis of an elution diagram, but subsequent analyses showed that the diagram was of small value in locating the desired UDPMurNAc. The pH of the three fractions was adjusted to 6.2 with LiOH, and the solutions were evaporated to dryness at 30° *in vacuo*. The syrupy residues were freed of chloride by dissolution in methanol and reprecipitation with acetone; at least five such operations were required. Total phosphate and MurNAc (after hydrolysis for 15 min at 100° in 0.01 N HCl) were determined.

On the basis of these analyses fraction II, which weighed 2.65 mg, contained 1.85 mg of UDPMurNAc in the form of the anhydrous trilithium salt, or 3.4% of the theoretical yield. This fraction was kept separately and was not further purified.

Fraction I contained an excess of ultraviolet-absorbing material over the amount of MurNAc present, and fraction III contained an excess of phosphorus over the quantity of MurNAc. Paper chromatography in ethanol–0.5 M ammonium acetate (pH 3.8) (5:2) of the three fractions revealed in each of them two nucleotide-phosphate substances, of which the faster one, with an R_F of 0.29, contained MurNAc.

To purify and isolate the UDPMurNAc in fractions I and III, the solids were recombined, dissolved in 10 ml of water, and agitated for 1 hr with 1 g of acid-washed charcoal. The supernatant was treated again with 200 and 300 mg of additional charcoal. After these treatments the ultraviolet-absorbing material was completely adsorbed. The charcoal was washed with five 25-ml portions of H_2O by suspension for 1 hr and sedimentation, then eluted with eleven 10-ml portions of 50% ethanol containing 0.1% v/v of concentrated ammonia, and finally with five portions of 75% ethanol containing 0.1% ammonia.

The water washings contained acetylhexosamine and a nucleotide that migrated like uridine 5′-phosphate in solvent A. The 50% alcoholic eluates were found by chromatography to contain only one ultraviolet-absorbing substance. They were combined, concentrated to dryness, taken up in a small volume of methanol, and precipitated with acetone. The resulting colorless and amorphous product was dried *in vacuo* over P_2O_5 with gentle warming. The quantity (5.50 mg) represents 7.31 μmoles, approximately 10% of the theoretical maximum yield. Analytical data for the substance, uridinediphospho-*N*-acetylmuramic acid trilithium salt, are summarized in Table I.

On chromatography in solvent A the substance gave a single ultraviolet-absorbing spot with R_F 0.21. Standards of uridine 5′-phosphate, uridine, uridine 5′-phosphoromorpholidate, and uracil moved at R_F 0.14, 0.61, 0.49, and 0.64, respectively.

At room temperature in 0.01 N HCl, *N*-acetylmuramic acid was liberated at an imperceptible rate during 0.5 hr of observation and barely noticeable after 2 hr, but after 15 min at 100° the liberation was quantitative (see Figure 1).

Enzymatic Synthesis of UDPMurNAc-L[^{14}C]Ala. ENZYME PREPARATION. Cultures of *S. pyogenes* S_{23}, type 14, in logarithmic growth (6 and 10 hr) or at the plateau (18 hr) served as starting material; little difference was seen among various growth stages or between freshly harvested and frozen cells.

Cells were sedimented at 0° and 8000*g*, resuspended in water which, in the case of log cells, contained a small amount of hyaluronidase, agitated for 15 min, resedimented at 35,000*g*, and washed once again with water. The final pellets were frozen and stored, or used immediately.

Cells were disrupted by grinding of the cell paste with twice its weight of acid-washed ground glass in a precooled mortar in the cold room. The paste was transferred to a centrifuge tube with 0.02 M phosphate (pH 7.2–7.8) containing 0.005 M thioethanol; 2 ml of buffer was used/g of cells. The solids were sedimented at 0° and 8000*g* for 20 min, and the supernatant was removed and inspected for absence of unbroken cells. The supernatant contained 20–25 mg/ml of protein (Lowry method) which was not affected by longer or more vigorous centrifugation.

The supernatant was treated with three volumes of saturated ammonium sulfate (pH 7.2) containing 10^{-4} M EDTA, and was centrifuged for 10 min at 35,000*g*. The pellet was reconstituted to the original volume with 0.02 M phosphate (pH 7.2); the resultant solution served as crude enzyme preparation and contained 15–20 mg/ml of protein. It was used freshly made or after frozen storage.

The optimal quantity of protein for alanine incorporation was found to be 100–200 μg of protein/100-μl reaction mixture, which also contained the following additives: $MnCl_2$ (100 mμmoles), ATP (200 mμmoles), K_2HPO_4 (800 mμmoles), and Tris-HCl (pH 8.35, 4 μmoles). [^{14}C]Alanine and UDPMurNAc were incorporated as specified in Table III. Incubation at 37° was performed for 30 min.

The reaction was terminated by addition of 1 ml of

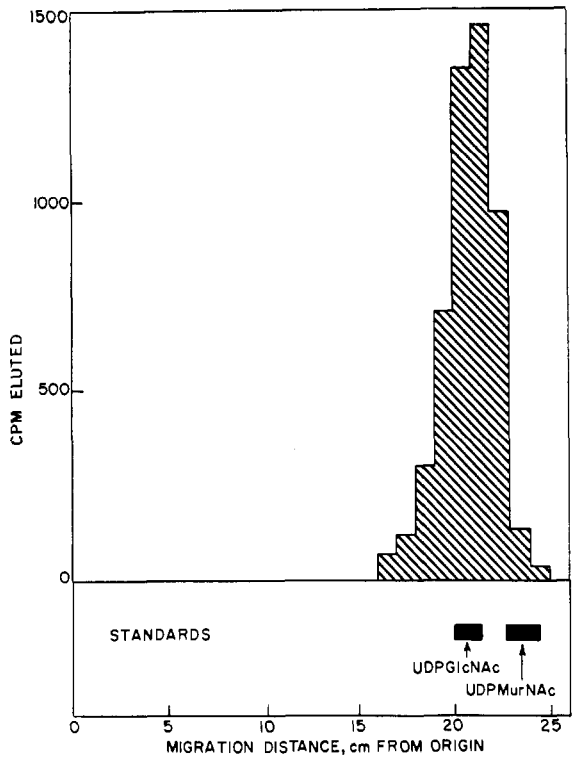

FIGURE 2: High-voltage electrophoresis of UDPMurNAc-[^{14}C]Ala. The separation was done on acid-washed Whatman No. 1 paper impregnated with 0.14 M sodium acetate buffer (pH 4.5) containing 0.65 g/l. of EDTA. The apparatus was supplied by Savant Instruments, Inc., New York. The potential gradient was 60 V/cm applied for 40 min.

cold 5% trichloroacetic acid. Protein was removed by centrifugation. Nucleotides were adsorbed by addition of 0.2 ml of a 5% suspension of Norit A, that had been washed twice with 1 N HCOOH, twice with EtOH–H_2O–NH_3 (5:4:1), and with water until neutral. Adsorption was allowed to proceed for 15 min with continuous agitation. The charcoal was sedimented at 27,000*g* at 0° for 10 min and washed with five 1-ml portions of 0.1 M NH_4OAc and once with cold H_2O. No radioactivity was detectable in the final wash liquids. Nucleotides were eluted with two 1-ml portions of 0.05 M NH_3 in 50% EtOH, and radioactivity of the eluates was determined with a Packard Tri-Carb liquid scintillation spectrometer.

Incorporation of radioactivity into the nucleotide fraction was determined as a function of enzyme protein concentration or of alanine to receptor ratio in numerous experiments. Of these we are reporting the one summarized in Table III, which demonstrates that with increasing values of that ratio, a greater and greater fraction of the receptor, UDPMurNAc, reacted with the alanine up to a maximum of 45–50%.

Characterization of UDPMurNAc-[^{14}C]Alanine. A charcoal eluate from an incubation of the type just described and containing radioactivity of 4450 cpm was chromatographed in solvent A on Whatman No. 1 paper, with UDPGlcNAc serving as a guide spot since this substance is reported as migrating like UDPMurNAc and UDPMurNAc-Ala. A spot derived from an enzyme incubation to which no UDPMurNAc had been added was likewise included as a control. Elution of appropriate areas of the chromatogram yielded 3700 cpm in the expected location. The eluates (sample and control) were lyophilized, hydrolyzed with 50 μl of 4 N HCl at 100° for 4 hr, and concentrated to dryness.

The residues were applied to a thin-layer chromatography plate of silica gel G and chromatographed with *n*-propyl alcohol–concentrated ammonia (7:3). After thorough drying, a radioautogram was prepared by allowing 2.5 days of contact time. Ninhydrin revealed spots in the positions of alanine and of muramic acid, as well as three or four other spots which also occurred in the control. Radioactivity was detected only in the alanine region of the hydrolysate stemming from UDPMurNAc-[^{14}C]alanine.

Pooled charcoal eluates from numerous incubations that had been performed in exploration of the parameters of the incorporation reaction were submitted to a lengthy process of purification. The main difficulty was the great similarity between UDPMurNAc and UDPMurNAcAla in chromatographic systems. Even the phenol solvent reported by Park and Johnson (1949) failed in our hands to give clear and reliable separation of the two nucleotides.

The procedure finally adopted employed segregation of the UDP-acetylhexosamine compounds in one area by paper chromatography in solvent A followed by high-voltage paper electrophoresis. At this point, the radioactivity was located in an area distinct from the location of UDPMurNAc. Figure 2 illustrates the results of the electrophoretic separation; UDPGlcNAc cannot be separated from UDPMurNAc-Ala but in the present study no need existed for such a separation. The final sample contained 96,000 cpm, corresponding to 40–50 mμmoles of alanine on the basis of the average specific activity of the [^{14}C]alanine samples used in incorporation studies. Impurities resulting undoubtedly from the filter paper, made it impossible to measure uridine by means of the absorption at 262 mμ, even when a blank paper correction was applied. The sample was dissolved in 300 μl of water. A 100-μl aliquot was found to contain 72 mμequiv of total phosphorus. The remaining 200 μl was adjusted with concentrated HCl to give 4 N acid and heated at 100° for 4 hr. After evaporation of the acid, the residue was applied to a column 0.9 × 60 cm of Bio-Rad A-4 resin and elution was performed at 50° with 0.2 N citrate buffer (pH 3.25). From previous experiments we knew that the facilities available to use were inadequate to detect the quantity of muramic acid to be expected. At the elution volume expected for alanine, ninhydrin-positive material emerged, and was radioactive. The quantity of alanine, corrected on the basis of a recovery experiment with a comparable mixture, was 43 mμmoles. Thus the ratio of P:Ala was found to be 2:1.2.

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Isolation and Characterization of 5′-*S*-Methyl-5′-thioadenosine from *Escherichia coli**

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ABSTRACT: A naturally occurring sulfur-containing nucleoside, 5′-*S*-methyl-5′-thioadenosine, was isolated from *Escherichia coli* B which was grown in a medium consisting of inorganic salts and glucose without the addition of methionine. The nucleoside was found only in the *E. coli* cells, in a concentration of 0.38 μmole/g of dry cells. The chemical and physical properties of this ^{35}S-labeled nucleoside and its derivatives were studied using gas, thin-layer, and paper chromatography, paper electrophoresis, and mass and ultraviolet spectroscopy. Microgram quantities of this nucleoside were isolated by means of thin-layer chromatography (solvent system, $CHCl_3$–CH_3OH–H_2O, 65:25:4, v/v). This adenosine derivative has its ultraviolet absorption maximum at 259.5 mμ (pH 7 and 10) and at 257 mμ (pH 2).

It possesses a pK_a' value of 3.4. 5′-*S*-Methyl-5′-thioadenosine was synthesized, and it has chemical and spectral properties identical with those of the isolated compound.

The occurrence of 5′-*S*-methyl-5′-thioadenosine (MTA)[1] in yeast extracts was first reported by Mandel and Dunham (1912), and its structure has been well established by a number of investigators (Suzuki *et al.*, 1924; Levene and Sobotka, 1925; Satoh, 1953). The addition of methionine to yeast cultures resulted in significant increases in the amount of MTA that could be isolated from the yeast. It was subsequently shown that the isolation of MTA was totally or partially the result of heat decomposition of *S*-adenosylmethionine during its extraction by boiling water (Schlenk and DePalma, 1957). However, enzymatic formation of MTA was later demonstrated by Shapiro and Mather (1958), and an enzyme was isolated from

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[1] Abbreviation use that is not given in *Biochemistry 5*, 1445 (1966), is: MTA, 5′-*S*-methyl-5′-thioadenosine.