

Enzymic Synthesis of a Diamino Sugar Nucleotide by Extracts of Type XIV *Diplococcus pneumoniae*^{1,2}

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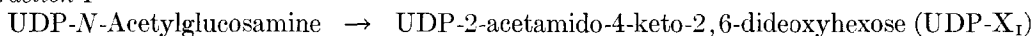
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We previously reported that incubation of the appropriate sugar nucleotides with extracts obtained from Type XIV *Diplococcus pneumoniae* resulted in the formation of glycolipids and serologically active polysaccharides (1, 2). During the course of these studies, we observed the formation of an unusual sugar nucleotide when the extracts were incubated with UDP-*N*-acetylglucosamine. As shown below, the new nucleotide is UDP-2-acetamido-4-amino-2,4,6-trideoxyhexose. The extracts also transferred the diamino sugar to endogenous lipid acceptors.

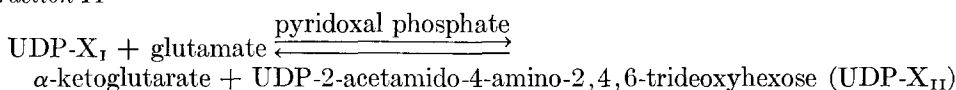
While diamino sugars are found in a few antibiotics (3), the only report of the occurrence of a 2,4-diamino-2,4,6-trideoxyhexose is that by Sharon and Jeanloz (4); the unusual amino sugar was a component of a polysaccharide produced by a strain of *Bacillus subtilis*. The amino sugar, called bacillosamine, was obtained as the 4-*N*-acetyl derivative after acid hydrolysis of the polysaccharide. Recently, Zehavi and Sharon (5) have identified bacillosamine as 2,4-diamino-2,4,6-trideoxy-*L*-altrose. Whether bacillosamine is identical to, or a diastereoisomer of the amino sugar reported below has not yet been established.

Two enzyme fractions were required for the synthesis of the UDP derivative of the diamino sugar. They catalyzed the following reactions:

Fraction I



Fraction II



These reactions are similar to those proposed for the enzymic synthesis of TDP-4-amino-4,6-dideoxyhexose from TDP-glucose (6), and perhaps for the biosynthesis of TDP-3-amino-3,6-dideoxyhexose (7).

¹ Dedicated to Luis F. Leloir on the occasion of his sixtieth birthday.

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MATERIALS AND METHODS

Diplococcus pneumoniae Type XIV ATCC 6314 was grown in Todd Hewitt Broth (Difco) as previously described (1, 8). When the culture had reached the late exponential phase of growth, it was chilled and the cells were harvested by centrifuging and washed with a solution contain-

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ing 0.15 M KCl, 0.01 M potassium phosphate buffer, pH 7.2.

Labeled and nonlabeled sugar nucleotides were chemically synthesized by a general method (9); UDP-*N*-acetylhexosamines-¹⁴C were labeled in the *N*-acetyl moieties.

Paper electrophoresis was performed at 60-70 V per centimeter for 15-20 minutes in the following buffers: 0.05 M sodium citrate, pH 4.5; 0.05 M pyridinium acetate, pH 6.5; and 0.03 M sodium phosphate, pH 7.5. Paper electrophoresis of labeled compounds, or of incubation mixtures containing labeled substrates, was followed by scanning the strips for radioactivity; the ¹⁴C was determined by cutting the strips into half-inch segments, which were then assayed in a Packard Tri-Carb liquid scintillation spectrometer. The toluene scintillation system recommended by the manufacturer was used in this assay.

The following solvent systems were employed for descending chromatography on Whatman No. 1 paper at room temperature: *I*, isobutyric acid-ammonia-water (57:4:39); *II*, 1% ammonium sulfate-isopropanol (1:2); *III*, 0.5 M ammonium acetate (pH 3.8)-ethanol (2:5); *IV*, 1.0 M ammonium acetate (pH 7.5)-ethanol (2:5); *V*, *n*-butanol-ethanol-water (4:1:1); and *VI*, ethyl acetate-pyridine-water (8:2:1). The mobilities of the enzymic products, their derivatives, and reference compounds are given in Table I. Kodak blue brand X-ray film was used for the detection of ¹⁴C compounds by radioautography. After chromatography, ultraviolet light-absorbing compounds were detected by examination under an ultraviolet light, carbohydrates with a periodate-benzidine dip reagent (10), and phosphate esters with the Hanes and Isherwood spray reagent (11), followed by ultraviolet light irradiation (12).

The following colorimetric methods were used: protein by the procedure of Lowry *et al.* (13), hexosamines by a modified Elson-Morgan method (14), reducing sugars by the method of Park and Johnson (15), and phosphate by the method of

Fiske and SubbaRow (16). Uridine was determined by its absorbance at 262 m μ in 0.075 M phosphate buffer, pH 7; an E_{\max} of 10,000 was used.

Periodate oxidation studies were conducted in the dark at 0°, and periodate consumption was measured titrimetrically with a micromethod (17). Acetaldehyde was removed from the periodate reaction mixtures by bubbling a stream of moist air through the solutions; the acetaldehyde was collected in 2% sodium bisulfite and measured by the procedure of Stotz (18). A chromotropic acid method was used to determine formaldehyde in the reaction mixtures (19).

Assay of enzymes. The incubation conditions used for assaying the enzymes are given in detail in the experiments presented below, and only the principles of the assay methods are discussed here. The general procedure involved the conversion of a labeled substrate to a labeled product, separation of these by paper electrophoresis, and determination of the product by liquid scintillation techniques.

The assay of Fraction I utilized a two-step method. In the first step, Fraction I was incubated with UDP-*N*-acetylglucosamine-¹⁴C, giving UDP-*X*_I-¹⁴C; the mixture was heated for 1 minute at 100° to stop the reaction. The second step involved the conversion of UDP-*X*_I-¹⁴C to UDP-*X*_{II}-¹⁴C by incubating the first reaction mixture with excess Fraction II, glutamate, and pyridoxal phosphate. Finally, the concentration of radioactive UDP-*X*_{II} was determined after paper electrophoresis in pyridinium acetate buffer. Under the conditions described in Fig. 1, at least 96% of UDP-*X*_I-¹⁴C added to boiled Fraction I in the first step was converted to UDP-*X*_{II}-¹⁴C. Control incubation mixtures, with heat-inactivated Fraction I, gave no detectable UDP-*X*_{II}-¹⁴C. The quantity of UDP-*X*_I formed in the reaction was proportional to the time of incubation and to the quantity of Fraction I employed.

A unit of Fraction I was defined as the quantity

TABLE I
R_F VALUES AND RELATIVE ELECTROPHORETIC MOBILITIES OF UDP-*X*_I AND UDP-*X*_{II}

Compound	<i>R_F</i> Values in solvents			Relative electrophoretic mobilities		
	I	II	III	Pyridinium acetate, pH 6.5	Citrate, pH 4.5	Phosphate, pH 7.5
UDP- <i>N</i> -Acetylglucosamine	.37	.33	.08	1.00	1.00	1.00
Uridine-5'-diphosphate	.37	.19	.04		1.29	1.47
Uridine-5'-monophosphate	.46	.33	.21	.86	.77	1.27
UDP- <i>X</i> _I	.37			1.00	1.00	
UDP- <i>X</i> _{II}	.46	.14	.07	.49	.44	.74
<i>N</i> -Acetylglucosamine 1-phosphate	.53	.33	.26		.81	

of enzyme(s) that converted 1 μ mole of UDP-*N*-acetylglucosamine to product in 30 minutes at 37° under the conditions described in Fig. 1.

Fraction II was assayed by measuring the reaction in the reverse direction since the quantity of UDP- X_I available for substrate was limited. Here the enzyme fraction was incubated with UDP- $X_{II-^{14}C}$, α -ketoglutarate, and pyridoxal phosphate; after stopping the reaction, the product, UDP- $X_I-^{14}C$, was separated from the substrate by electrophoresis and measured by liquid scintillation techniques. Under the conditions described below, the formation of UDP- $X_I-^{14}C$ was proportional to time of incubation and to the quantity of Fraction II.

A unit of Fraction II was defined as the quantity that converted 1 μ mole of UDP- X_{II} into UDP- X_I in 30 minutes.

Preparation of Fractions I and II. The following operations were conducted between 0° and 5°. The cells obtained from 6 liters of culture medium were suspended in 24 ml of the buffer-salts solution used for washing the cells, and ruptured with a French pressure cell. After stirring for several minutes with 2 mg crystalline DNase to reduce the viscosity, the suspension was diluted to 40 ml and centrifuged at 32,000 *g* for 1 hour. The translucent supernatant fluid contained the major portion of Fraction II, and was carefully separated from the particulate layer which contained both Fractions I and II. After decanting the upper layer, the tube was shaken to suspend the partially sedimented particulate fraction, and the latter was decanted. The tube was rinsed with 0.5-ml portions of the buffer-salts mixture, followed by careful decantation; a small quantity of packed residue remaining in the tube was discarded. The crude particulate fraction contained both Fractions I and II, and was used to prepare UDP- X_{II} as described below.

Fraction II could be removed from the particulate, leaving Fraction I, by storing the sediment at 0° for 2-5 days, followed by centrifuging at 32,000 *g* and washing the particles twice with fresh buffer-salts solution. The quantity of wash fluid used was equal to the volume of the original suspension of particles. The washed preparation was finally suspended in 4 ml of the buffer-salts solution; it contained 5 units of Fraction I per milliliter and 8.5 mg protein per milliliter. Fraction I was stable to storage at -15°, and at 0° for 1 week.

Fraction II was purified as follows. The crude supernatant fraction (35 ml) was titrated with 1% protamine sulfate solution (about 9 ml) until no further precipitate was formed. After 10 minutes with occasional stirring, the mixture was centrifuged at 15,000 *g* for 10 minutes, and the precipitate was discarded. The supernatant fluid (40 ml) was treated with 36 ml of a saturated solution of

ammonium sulfate. (The ammonium sulfate was prepared by saturating a solution at 5° and adjusting with concentrated ammonium hydroxide until a pH of 7 was obtained when the solution was diluted 1:10 with water). After the addition of ammonium sulfate, the mixture was stirred occasionally for 20 minutes at 0° and centrifuged for 10 minutes at 15,000 *g*, and the residue was discarded. This residue contained all of the Fraction I activity that remained in the crude supernatant fluid, and represented about 10% of the total Fraction I activity of the crude extract; it was not soluble when resuspended in fresh buffer-salts solution, i.e., it sedimented when centrifuged at 100,000 *g*. The supernatant fluid (73 ml) obtained after the first ammonium sulfate treatment contained all of the Fraction II activity, which was in turn precipitated by adding an additional 36 ml of the saturated ammonium sulfate solution. After occasional stirring for 15 minutes at 0°, followed by centrifugation for 10 minutes at 15,000 *g*, the precipitate was dissolved in 4 ml of buffer-salts solution and dialyzed for 12 hours against the same solution to remove ammonium sulfate. Finally, the dialyzed solution was centrifuged at 100,000 *g* to remove any possible contamination of Fraction II by Fraction I. A typical preparation contained 67 units of Fraction II activity, and 86 mg protein in a volume of 6.7 ml. The preparation was stable to storage at -15° for at least 3 weeks. Fraction II was purified approximately 10-fold over the crude extract.

RESULTS

Properties of Fraction I. Fractions I and II, obtained as described above, were crude preparations, and the results of kinetic experiments presented below are therefore considered preliminary.

The conversion of UDP-*N*-acetylglucosamine to UDP- X_I was proportional to time of incubation for at least 30 minutes, and to the quantity of Fraction I in the range 0.01-0.05 unit per incubation mixture.

The effect of pH on the rate of Reaction I is shown in Fig. 1. The optimum in Tris-HCl buffer was approximately pH 7.4. An apparent inhibition was observed in either phosphate or Tris-phosphate buffers.

The effect of substrate concentration on Reaction I is shown in Fig. 2, and according to these results, the K_m for UDP-*N*-acetylglucosamine was approximately 1×10^{-3} M; it should be noted, however, that the particulate fraction contained UDP-*N*-acetylglucosamine 4'-epimerase (1), which would

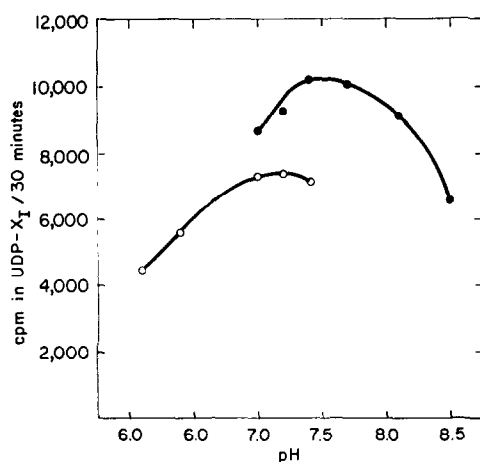


FIG. 1. The effect of pH on Fraction I activity. Incubation mixtures (50 μ l) contained 0.20 μ mole UDP-*N*-acetylglucosamine (1.8×10^5 cpm/ μ mole), 0.05 unit Fraction I, and 3.3 μ moles Tris-HCl (●) or potassium phosphate buffer (○). The mixture was incubated for 30 minutes at 37° and heated to 100° for 1 minute to stop the reaction. After cooling, the following additions were made: 25 μ l 0.1 M sodium glutamate; 25 μ l 0.33 M Tris-HCl buffer, pH 8; 5 μ l 0.005 M pyridoxal phosphate; and 25 μ l purified Fraction II (0.25 unit). The mixtures were incubated for 2 hours at 37° and assayed by the electrophoretic method.

reduce the concentration of the substrate, and the K_m is therefore only an estimate.

Figure 3 shows the effect of time of incubation on the reaction. The rate decreased after about 30 minutes, and the reaction essentially ceased after approximately 60 minutes. The explanation for the decrease in rate is apparently not enzyme inactivation, since the addition of fresh enzyme at 2 hours gave only a slight increase of UDP- X_I . Another possibility was that the reaction had reached its equilibrium value; this explanation was not valid since only negative results were obtained in attempts to show that the reaction catalyzed by Fraction I was reversible (Table II).

One of the reasons that the rate of Reaction I abruptly decreases may be inhibition of the reaction by the product. The inhibition experiments are given in Table II; UDP- X_I was a powerful inhibitor of Fraction I, and UDP- X_{II} also inhibited, but to a lesser extent.

Fraction I was neither stimulated nor in-

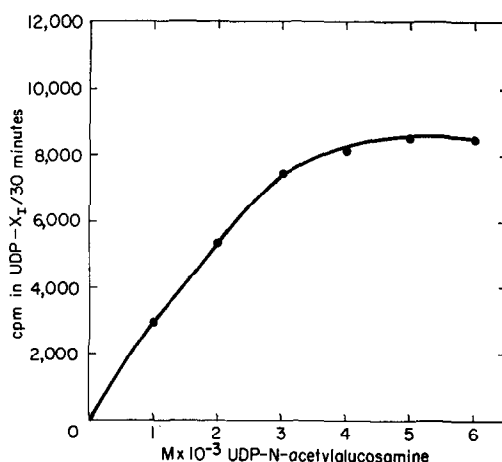


FIG. 2. The effect of substrate concentration on Reaction I. Incubation mixtures (50 μ l) contained: 3.3 μ moles Tris-HCl buffer, pH 7.4; 0.05 unit Fraction I; and UDP-*N*-acetylglucosamine (1.8×10^5 cpm/ μ mole). The reaction mixtures were treated as described in Fig. 1.

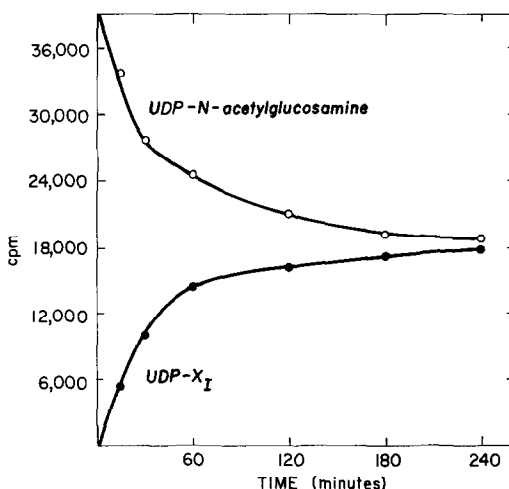


FIG. 3. Effect of time of incubation on Reaction I. Incubation conditions and assay were performed as indicated in Fig. 2. The upper curve represents the concentration of UDP-*N*-acetylglucosamine-¹⁴C in the reaction mixtures at the indicated times; the lower curve gives the concentration of UDP- X_I -¹⁴C.

hibited by concentrations of $MgCl_2$ ranging from 0.002 to 0.01 M. However, in the presence of 0.01 M EDTA, the enzymic activity was inhibited to the extent of 32%.

Substrate specificity studies indicated that the system was specific for UDP-*N*-

TABLE II

EFFECT OF REACTION PRODUCTS ON FRACTION I

Incubation mixtures (50 μ l) contained: 3.3 μ moles Tris-HCl buffer, pH 7.4; 0.05 unit Fraction I; and where indicated: UDP-*N*-acetylglucosamine (1.0×10^6 cpm/ μ mole), and UDP- X_I or UDP- X_{II} (4.4×10^4 cpm/ μ mole). The incubation mixtures were treated as described in Fig. 1 to convert UDP- X_I to UDP- X_{II} .

Additions (μ mole)			Incubation time with Fraction I (min)	UDP- X_{II} formed by Fraction II (cpm)
UDP- X_I	UDP- X_{II}	UDP- <i>N</i> -Acetylglucosamine		
0.15			0	6,120
0.15			30	6,040
0.15			120	6,310
0.10		0.20	30	28,700
0.10		0.10	30	22,700
	0.10	0.20	30	49,500
	0.10	0.10	30	38,600
		0.20	30	55,100
		0.10	30	42,400

acetylglucosamine. A nucleotide analogous to UDP- X_{II} was not formed when the complete system was incubated with UDP-glucose, and a sugar phosphate analogous to the phosphate ester of X_{II} was not formed when *N*-acetylglucosamine-1-P was substituted for UDP-*N*-acetylglucosamine. On the other hand, UDP-*N*-acetylgalactosamine could apparently replace UDP-*N*-acetylglucosamine. Further investigation showed, however, that this apparent activity resulted from the contaminating 4'-epimerase. For example, the quantity of UDP- X_{II} formed from UDP-*N*-acetylgalactosamine was 9% of that obtained from UDP-*N*-acetylglucosamine when the incubation was conducted for 15 minutes, and 34% after 30 minutes.

Properties of Fraction II. Under the conditions described in Fig. 4, the quantity of UDP- X_I formed from UDP- X_{II} was proportional to the time of incubation for at least 30 minutes, and to the quantity of Fraction II present in the incubation mixture. As shown in the figure, the pH optimum in Tris-HCl buffer was about 7.9.

The enzymic activity was not affected by the addition of either 0.01 M MgCl₂ or EDTA. On the other hand, when pyridoxal phosphate was omitted, no activity could be detected.

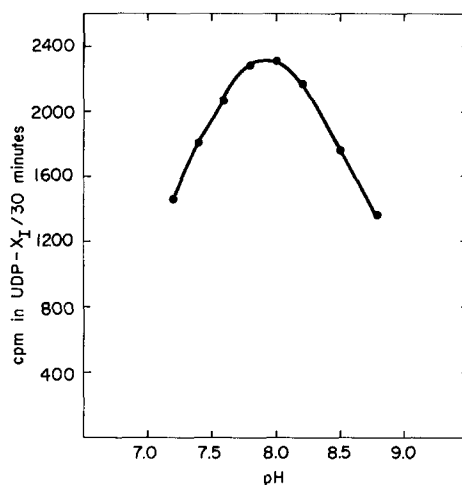


FIG. 4. The effect of pH on Reaction II. Incubation mixtures (75 μ l) contained: 0.1 μ mole UDP- X_{II} (4.4×10^4 cpm/ μ mole); 2 μ moles sodium α -ketoglutarate; 8 μ moles Tris-HCl buffer; 0.01 μ mole pyridoxal phosphate; and 0.05 unit Fraction II. The mixture was incubated at 37° for 30 minutes, the reaction was stopped by heating at 100° for 1 minute, and the ¹⁴C-products were determined by the electrophoretic method.

The presence of α -ketoglutarate was also essential for enzymic activity with purified Fraction II, and this compound could not be replaced by equimolar concentrations of sodium oxalacetate, pyruvate, or glyoxalate. When the complete system was used, i.e., the crude particulate preparation, glutamate was active and could not be replaced by aspartate or asparagine, and was only partially replaced by glutamine. In contrast to the reaction(s) catalyzed by Fraction I, under the proper conditions, the transaminase reaction catalyzed by Fraction II resulted in the quantitative conversion of the substrate, UDP- X_{II} , to UDP- X_I (Fig. 5).

Preparation of UDP- X_{II} . The crude particulate preparation described above, containing Fractions I and II, was used as a convenient enzyme system for the preparation of UDP- X_{II} . Attempts to conduct these experiments on a large scale gave low yields of the product. The incubations were therefore conducted on a small scale, and several such mixtures were combined. A typical reaction mixture contained the following components in a final volume of 10 ml: 4 ml of the crude particulate preparation; 20

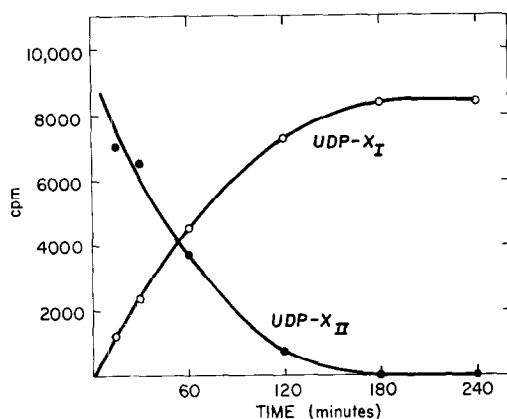


FIG. 5. The effect of time of incubation on Reaction II. Incubation mixtures were prepared as described in Fig. 4, at pH 7.9. The curves represent the quantities of UDP-X_I and UDP-X_{II} (both ¹⁴C) present in the incubation mixtures at the indicated times.

μ moles of UDP-*N*-acetylglucosamine (labeled in the carboxyl group of the *N*-acetyl moiety, specific activity 5.5×10^4 cpm per micromole); 100 μ moles sodium glutamate; and 0.5 μ mole pyridoxal phosphate. The pH of the mixture was 7.4, and it was incubated at 37° for 4 hours. Assay of aliquots showed that 5–7 μ moles of labeled UDP-X_{II} were formed during the incubation; the mixture was centrifuged at 32,000*g* for 1 hour, and the supernatant fluid was stored at –15°.

When approximately 100 μ moles of UDP-X_{II} had been accumulated, the solutions were combined and filtered through a 2 \times 15 cm column of SGL charcoal.⁴ After washing the charcoal with water, the nucleotides were eluted with 100 ml of a solution containing 50% ethanol and 0.6% NH₃. The eluate was concentrated to dryness in a vacuum, the residue was dissolved in 2 ml of water, and the solution was subjected to paper electrophoresis on 30 sheets of Whatman 3 MM paper (15 inches wide each) in the

⁴The SGL charcoal, coarse mesh, was obtained from the Pittsburgh Chemical Co. It was prepared for use by repeated treatment with approximately 1 *N* HCl, washed with water, and then washed extensively with 50% ethanol containing 0.6% NH₃, and finally with water. The ethanol-ammonium hydroxide wash was important since it removed ultraviolet-absorbing and lipid-like materials from the charcoal.

pyridinium acetate buffer described above. The segments of the paper containing ¹⁴C were eluted with water. The primary reason for the electrophoresis step was the fact that it removed inorganic phosphate, a contaminant that was difficult to remove in the subsequent steps.

The eluate from the paper was concentrated in a vacuum and applied to a 2 \times 25 cm column of Dowex-1, bicarbonate-form resin (200–400 mesh). After being washed in water, the column was eluted with a linear gradient of NH₄HCO₃; the 4-liter gradient ranged from 0.0 to 0.5 *M* NH₄HCO₃. Two radioactive peaks were detected when aliquots of the 20-ml fractions were assayed; Peak I was observed in fractions 68–100, and Peak II was detected in fractions 106–138. Paper electrophoresis indicated that Peak I was UDP-*N*-acetylglucosamine-¹⁴C, and Peak II was UDP-X_{II}-¹⁴C; these results were surprising in view of the mobilities of these substances on paper electrophoresis. Peak II was treated in a vacuum with an excess of Dowex-50, hydrogen-form resin, to remove the NH₄HCO₃. After filtration, the solution was neutralized with NH₄OH, concentrated in a vacuum, and applied to a 2 \times 25 cm column of Dowex-1, chloride-form resin (200–400 mesh). The column was first washed with 0.003 *M* HCl to remove small amounts of ultraviolet light-absorbing materials, and then eluted with 1500 ml of 0.005 *M* LiCl in 0.003 *N* HCl. The fractions containing UDP-X_{II} were combined, adjusted to pH 7 with LiOH, and concentrated to dryness in a vacuum. Finally, the solid was dissolved in 3 ml of methanol, the UDP-X_{II} was precipitated by the addition of 37 ml of acetone, and the precipitate was collected by centrifugation, dissolved in methanol, again precipitated with acetone, and collected by centrifugation. The resulting white powder was dried over calcium chloride, in a vacuum, for 24 hours; it weighed 49 mg and was obtained in 75% yield.

Properties of UDP-X_{II}-¹⁴C. The product obtained as described above was homogeneous when examined by paper chromatography (solvent systems I, II, III, and IV), and paper electrophoresis (3 pH's). The ultraviolet light absorption spectrum of UDP-X_{II} corresponded exactly to that of

UMP at pH 2, 7, and 9.5. Different preparations showed only traces of inorganic phosphate, while the organic P to uridine molar ratio was 1.87. On a weight basis, the uridine content indicated that the molecular weight of the product was 754. The specific radioactivity, 4.4×10^4 cpm per micromole of uridine, was lower than that of the substrate, 5.5×10^4 .

The presence of a free amino group in UDP- X_{II} , in addition to the *N*-acetyl- ^{14}C group, was shown by the following: (a) The product reacted with ninhydrin on the paper chromatograms. (b) Under the standard conditions used for *N*-acetylation of hexosamines (20), the product was converted to a substance that migrated on paper electrophoresis in the same manner as UDP-*N*-acetylglucosamine. The labeled acetyl group was retained during this procedure. (c) When UDP- X_{II} was treated with 2,4-dinitrofluorobenzene (21), a compound was formed that migrated on paper electrophoresis more rapidly than UDP- X_{II} ; the yellow dinitrophenyl spot corresponded to the radioactive spot (Fig. 6).

Quantitative estimation of the reaction with 2,4-dinitrofluorobenzene showed that approximately one mole of the reagent reacted per mole of uridine, thus indicating that the UDP- X_{II} contained one free amino group. It was possible that this group was derived from the amino group of the *N*-acetylglucosamine residue by an acyl migration, thereby retaining the ^{14}C in the product, but as an acetyl ester. However, this appeared unlikely when we observed that the acetyl group was completely stable to alkali under conditions that would quantitatively hydrolyze *O*-acetyl esters.

An important property of UDP- X_{II} was its lability to acid. For example, when treated with 0.1 *N* HCl for 10 minutes at 100°, the colorless solution of UDP- X_{II} quickly turned red and then darkened with the formation of considerable humin. Examination of the mixture by paper electrophoresis showed UDP as the major identifiable substance, with small amounts of UMP, and a little of the original nucleotide. The hydrolysis of UDP- X_{II} gave one mole of NH_3 (Fig. 7). This decomposition, and the formation of the humin, could

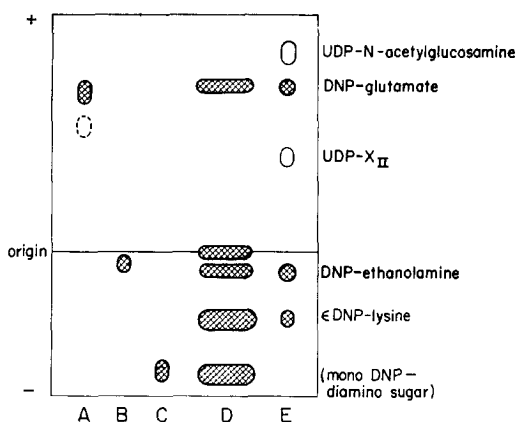


FIG. 6. Electrophoresis of DNP-derivatives of the diamino-sugars in citrate buffer, pH 4.5. *A*; UDP- X_{II} -DNP, prepared as follows: *A* mixture containing 50 μ l 0.01 *M* UDP- X_{II} , 50 μ l 5% (v/v) triethylamine, and 200 μ l 5% 2,4-dinitrofluorobenzene in ethyl alcohol was placed in the dark for 3 hours. The solvent was removed in a vacuum, 0.25 ml 5% triethylamine was added, and the solution was extracted with ether 4-5 times. The aqueous phase was subjected to electrophoresis. *B*; the product in *A* was hydrolyzed in 0.1 *N* HCl at 100° for 15 minutes, and the HCl was removed in a vacuum over $CaCl_2$ and soda lime. Water was added and the mixture was subjected to electrophoresis. *C*; the same as *B* except that hydrolysis was performed in 2 *N* HCl for 2 hours. *D*; the DNP derivative of "*C*" polysaccharide was prepared as described in *A* and hydrolyzed at 100° in 2 *N* HCl for 2 hours. The hydrolysate was dried and electrophoresis was performed as above. *E*; reference compounds. Cross-hatching indicates yellow color on the paper.

largely be prevented when the acid treatment was conducted with the derivatives formed by *N*-acetylation or by dinitrophenylation. Even here, however, vigorous acid hydrolysis resulted in decomposition.

The decomposition of UDP- X_{II} under acid conditions suggested the possibility that pyrroles were being formed, and this possibility was tested with the Ehrlich reagent (24). A positive test was obtained; when the reaction was conducted at 80° for 5 minutes (Table III), the absorption maximum was at 550 $m\mu$, and the molar extinction coefficient (based on uridine) was 7.6×10^3 . The optimum conditions for performing the direct Ehrlich reaction on this substance have not been established; the color was not stable to

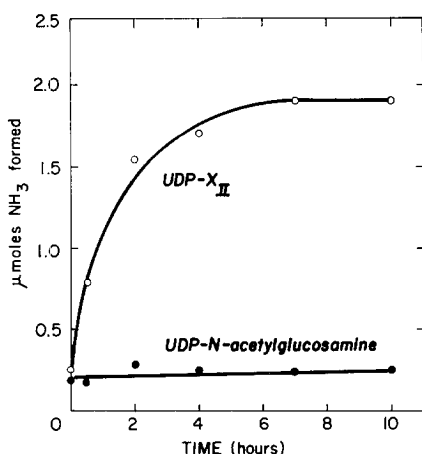
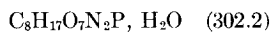


FIG. 7. The liberation of NH_3 during hydrolysis of UDP-X_{II} . Hydrolysis mixtures (1.3 ml) contained 2.14 μmoles of UDP-X_{II} or 2.0 μmoles $\text{UDP-N-acetylglucosamine}$ in 2 N HCl. The mixtures were heated at 100° and aliquots were removed at various times. Ammonia was determined in the aliquots by the Conway diffusion technique (22) followed by nesslerization (23). A standard solution of $(\text{NH}_4)_2\text{SO}_4$ treated in the same manner gave quantitative yields of NH_3 by this procedure.

longer periods of heating. The application of the Ehrlich reaction to the analysis of X_{II} is discussed below. UDP-X_{II} also gave a positive reaction with the resorcinol reagent (25); the absorption maximum was at 590 $\text{m}\mu$, and the molar extinction coefficient was 2.3×10^3 . As with the Ehrlich reagent (discussed below), the resorcinol reagent gave no color with X_{II} after reduction with sodium borohydride, and considerably less color with the acetyl derivative of X_{II} (obtained by *N*-acetylation with acetic anhydride).

Isolation and properties of the phosphate ester of X_{II} . Preliminary experiments showed that UDP-X_{II} was hydrolyzed by venom phosphodiesterase to UMP (identified by chromatography in solvent systems I, II, III, and IV), and to a radioactive compound that migrated very slowly on paper electrophoresis in citrate or pyridinium acetate buffers. The ^{14}C product was isolated from large-scale incubation mixtures containing the following components in final volumes of 5 ml: 75 μmoles $\text{UDP-X}_{\text{II}}\text{-}^{14}\text{C}$; 50 μmoles MgCl_2 ; 175 μmoles Tris-HCl buffer, pH 7.8; and 600 units of venom phosphodiesterase

(California Biochemical Corp.). After 1 hour at 37° , electrophoresis of an aliquot showed that 80% of the substrate had been converted to a product that remained close to the origin. The product was isolated by a procedure used for the purification of other hexosamine phosphates (26); the reaction mixture was applied to a column containing 500 ml of Dowex-50, hydrogen-form resin (200–400 mesh), and eluted with water. A small fraction of the total radioactivity was eluted with the salts in the first 400 ml, and the bulk of the radioactivity was eluted in a peak emerging between 4350 and 4850 ml of eluant. The latter was evaporated to dryness in a vacuum, dissolved in 0.5 ml of water, and treated with 1.5 ml of ethanol and 1.5 ml of acetone. After storage at 5° , the product crystallized as fine needles which were collected by centrifugation, washed with an ethanol-acetone mixture (1:1), and dried over calcium chloride in a vacuum. The product weighed 10 mg and was recrystallized from a water-ethanol-acetone mixture. After drying over P_2O_5 in a vacuum for 24 hours, it exhibited $[\alpha]_D^{25} = +126^\circ$ (*c*, 0.3 in water), and gave the following elemental analyses (Spang Microanalytical Laboratory, Ann Arbor, Michigan).



Calculated: C 31.79 H 6.34 N 9.27 P 10.26

Found: C 31.77 H 6.28 N 9.30 P 10.36

The crystalline product did not give a reducing sugar test but reacted with ninhydrin on paper chromatograms.

*Preparation and Reduction of X_{II} and of *N*-acetyl- X_{II} .* X_{II} , its *N*-acetyl derivative, and the corresponding sugar alcohols were prepared from $\text{X}_{\text{II}}\text{-1-P}$. Since the latter compound could not be hydrolyzed with acid without concomitant destruction, the phosphate group was removed by enzymic hydrolysis. Treatment of $\text{X}_{\text{II}}\text{-1-P}$ (6 μmoles adjusted to pH 8 with NaOH) with 0.1 mg *Escherichia coli* alkaline phosphatase (Worthington Biochemical Corp.) for 1–3 hours at 37° gave a product that showed a positive charge on paper electrophoresis in citrate buffer. The reaction was conducted until the phosphate ester was completely hydrolyzed. At this point, aliquots were directly *N*-acetylated with acetic anhydride

TABLE III
 COLORIMETRIC ANALYSIS OF THE DIAMINO SUGARS

Compound	Amount analyzed (μ moles)	Direct Ehrlich's ^a (absorbance)	Dinitrophenylation ^b (absorbance)
UDP-X _{II}	0.022	0.149	0.069
	0.044	0.275	0.149
	0.11	0.625	0.361
	0.22	1.380	0.705
UDP-X _{II}	0.10	0.66	
X _{II} (free sugar)	0.10	1.81	
<i>N</i> -Acetyl-X _{II} (free sugar)	0.10	1.24	0.00
UDP- <i>N</i> -Acetyl-X _{II}	0.10	0.361	0.00
NaBH ₄ reduced X _{II}	0.10	0.00	
NaBH ₄ reduced <i>N</i> -acetyl-X _{II}	0.10	0.00	
"C" polysaccharide ^c	0.50 mg	0.205	
	0.25 mg	0.102	0.133
	0.125 mg		0.062
SXIV polysaccharide ^d	0.50 mg	0.00	0.00

^a An aqueous solution (0.1 ml) containing the substance to be analyzed, 0.9 ml ethyl alcohol, and 0.2 ml Ehrlich's reagent (0.5% *p*-dimethylaminobenzaldehyde in 1:1 concentrated HCl-ethyl alcohol) was heated at 80° for 5 minutes and cooled in tap water. The absorbance of the solution was determined at 550 $m\mu$ after 30 minutes.

^b The DNP derivative of the substance to be analyzed was prepared as described in Fig. 6C. After preparation of the derivative, the samples were hydrolyzed in 2 *N* HCl at 100° for 2 hours. The solution was evaporated over CaCl₂ and soda lime, 0.1 ml water was added, and 0.05 ml was subjected to electrophoresis (citrate buffer). After drying the paper, the yellow spots corresponding to DNP-diamino sugar were suspended in 2 ml 0.01 *N* HCl for 4 hours, and the absorbancies of the solution were determined at 360 $m\mu$.

^c Generously supplied by Dr. Emil Gotschlich (Rockefeller Institute).

^d Kindly provided by Dr. Michael Heidelberger (New York University).

(20), or were reduced with sodium borohydride, or were first *N*-acetylated and then reduced. These procedures gave reduced X_{II} and reduced *N*-acetyl-X_{II}. The borohydride reductions were performed by adding a 100-fold excess of NaBH₄ to the reaction mixtures over a period of 30 minutes. The excess borohydride was destroyed by adding HCl to pH 4, and the mixtures were passed through a column of Dowex-1, chloride-form resin, to remove borate ion. The eluates were concentrated in a vacuum to 0.5 ml and treated with 5 volumes of ethanol to precipitate the bulk of the NaCl. After filtration, the salt was washed with ethanol, the filtrates were concentrated, and the products were purified by chromatography on Whatman No. 1 paper in solvent system V; the labeled products were detected by radioautography. The sugar alcohol derived from X_{II} migrated with an $R_F = 0.29$, while the

R_F of the *N*-acetyl derivative was 0.50. The products were eluted from the paper with water and oxidized with sodium periodate. As shown in Table IV, the alcohol corresponding to X_{II} consumed 2 moles of periodate, gave 0.86 mole of acetaldehyde, and no detectable formaldehyde. By contrast, the alcohol corresponding to *N*-acetyl-X_{II} consumed less than 0.2 mole of periodate and gave no detectable acetaldehyde or formaldehyde. The eluate of a control segment of the chromatography paper per se did not consume significant quantities of periodate.

The sugar alcohols obtained from X_{II} and *N*-acetyl-X_{II} were radioactive and therefore contained the labeled *N*-acetyl group originally present in the UDP-*N*-acetylglucosamine-¹⁴C. Since this acetamido group was located at C-2 of the hexosamine, the results of the periodate oxidation studies with the sugar alcohols are only consistent

TABLE IV
PERIODATE STUDIES WITH THE DIAMINO SUGARS^a

Oxidation time	NaBH ₄ reduced X _{II} (moles IO ₄ ⁻ /mole sugar)	NaBH ₄ reduced <i>N</i> - acetyl-X _{II} (moles IO ₄ ⁻ /mole sugar)
15 minutes	1.37	0.12
30 minutes	1.58	0.14
2 hours	2.10	0.14
4 hours	2.01	0.20
8 hours	2.06	0.17

^a The reaction mixtures contained the following components in 1 ml: 1.90 μ moles reduced X_{II} or 2 μ moles reduced *N*-acetyl-X_{II}, determined by ¹⁴C-content; 20 μ moles sodium periodate; and 200 μ moles acetate buffer, pH 4.5. The reaction mixtures were maintained at 0° in the dark, and aliquots were removed for analysis. The values represent periodate consumption per mole of sugar, and these were determined by a micro-titrimetric method utilizing arsenite (17). Other reaction mixtures were analyzed for aldehydes as described in *Methods*. Acetaldehyde was obtained on periodate treatment of reduced X_{II} (0.86 mole per mole ¹⁴C-sugar) but not from reduced *N*-acetyl-X_{II}. Formaldehyde was not obtained as a product of periodate oxidation of either sugar.

with a 6-deoxy group at C-6 (which gave acetaldehyde), and with an amino function at C-4. The periodate oxidation studies, along with the other properties of UDP-X_{II} and the crystalline X_{II}-phosphate described above, showed that X_{II} was a 2-acetamido-4-amino-2,4,6-trideoxyhexose. The configuration of the sugar has not yet been established, and its possible relationship to bacillosamine has been discussed above.

Preparation of UDP-X_I. The most convenient method for the preparation of this nucleotide was from UDP-X_{II}, using Fraction II. When the incubation was conducted on a large scale (10 μ moles of UDP-X_{II}) as described in Fig. 5, for 2 hours, essentially all of the UDP-X_{II} was converted to UDP-X_I. The latter was isolated after electrophoresis of the mixture in pyridinium acetate buffer, and the eluate from the paper was applied to a 1 \times 10 cm Dowex-1, chloride-form column (200–400 mesh). After washing the column with water, it was eluted with 0.003 *N* HCl in a linear gradient of LiCl, ranging from 0.0 to 0.08 *M*, in a total volume of 1 liter. The fractions, 10 ml each, were assayed for

nucleotide and for ¹⁴C; only one peak was detected, in fractions 61–80. The appropriate fractions were combined, the solution was filtered through a 5-ml column containing SGL coarse mesh charcoal,⁴ the charcoal was washed with water, and the nucleotide was eluted with ethanol-water-ammonia as described above. The product was finally obtained by concentrating the eluate to dryness in a vacuum.

The product, UDP-X_I, was a substrate for Fraction II, being converted in 96% yield to UDP-X_{II} upon incubation with the transaminase, in the presence of pyridoxal phosphate and excess glutamate. The ultraviolet absorption spectrum of the nucleotide at pH 7 was characteristic of UMP, but also showed "end absorption" in the range below 230 *m* μ . The end-absorbing substance(s) was not removed by an additional purification by paper electrophoresis. Furthermore, the ratio of ultraviolet light absorbancy to ¹⁴C was about 12% higher than that of the substrate, UDP-X_{II}. These results suggest an impurity that was not removed by the purification procedures, or that the carbonyl group may contribute significantly to the absorption spectrum.

The nucleotide contained a carbonyl function as indicated by the following: (a) It reacted with *o*-phenylenediamine (27), and the reaction product showed the expected light absorption spectrum with a molar extinction coefficient at 335 *m* μ of 5.2×10^3 . (b) The nucleotide gave a positive reducing sugar test (15) without prior acid hydrolysis. (c) After hydrolysis with acid, a negative hexosamine reaction was obtained with the Elson-Morgan reagents. However, if the nucleotide was first reduced with sodium borohydride, and then subjected to this procedure, the Elson-Morgan test was positive.

The hexosamine(s) from UDP-X_I (2 μ moles) was prepared as follows. UDP-X_I (2 μ moles) was reduced with sodium borohydride, adsorbed on charcoal, eluted, and hydrolyzed in 4 *N* HCl at 100° for 4 hours. The hydrolyzate was dried over soda lime and calcium chloride to remove the HCl, and the residue was chromatographed by the procedure of Gardell (28). The results shown in Fig. 8 clearly showed that the products obtained from UDP-X_I were neither glucosamine nor

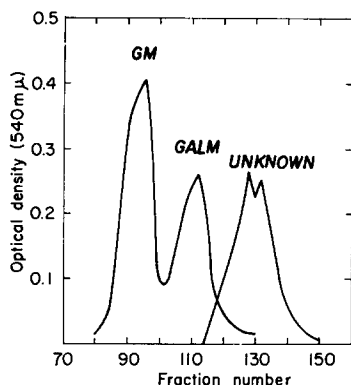


FIG. 8. Chromatography of the hexosamines from reduced UDP- X_I on Dowex-50 (H^+). The hexosamines obtained from 1 μ mole UDP- X_I were chromatographed by the procedure of Gardell (28). The column was 1 \times 46 cm, 200-400 mesh, Dowex-50 (H^+); the eluant was 0.3 N HCl, and 1.78-ml fractions were collected. Glucosamine and galactosamine standards were chromatographed in the same manner. Alternate fractions were analyzed for hexosamine after drying over $CaCl_2$ and soda lime.

galactosamine. The hexosamine fractions were combined, concentrated, and degraded with ninhydrin by the procedure of Stoffyn and Jeanloz (29). The degraded sugar(s) migrated more rapidly than standard pentoses: in solvent system V, the R_{ribose} was 1.81, while in solvent system VI, the R_{ribose} was 2.51. These results agreed with those obtained by ion-exchange chromatography of the hexosamine(s), and would be expected if the hexosamines derived from X_I were the 2-amino-2,6-dideoxyhexoses.

The characterization of UDP- X_I has not been completed. Nevertheless, all of the evidence presented above, i.e., its origin from UDP- X_{II} by a reaction that shows all of the properties of a transamination, the fact that it contains a carbonyl group, that it gives hexosamines on reduction which are not glucosamine or galactosamine, and that these hexosamines are not degraded by ninhydrin to known pentoses but to substances that appear to be less polar, suggest that X_I is a 4-keto-2-acetamido-2,6-dideoxyhexose. The origin of this compound from UDP-*N*-acetylglucosamine would be analogous to the reactions involving the conversion of several hexose nucleotides to the corresponding 4-keto-6-deoxyhexose nucleotides (30), the

latter being intermediates in the formation of substances such as GDP-L-fucose.

Detection of X_{II} in biological materials. As emphasized above, the diamino sugar, X_{II} , rapidly decomposes in acid. It therefore appears possible that this or similar substances may occur in biological substances, but have hitherto escaped detection with the exception of bacillosamine (4). The direct Ehrlich reaction, which probably depends upon the conversion of X_{II} to pyrroles (ring closure would involve the C-4 amino and C-1 aldehyde groups), can be used to detect X_{II} , but this reaction would be dependent on X_{II} glycosides being sensitive to hydrolysis so that cleavage could occur under the relatively mild acid conditions used in the Ehrlich test. In fact, the free amino sugar, X_{II} , obtained by the enzymic hydrolytic methods described above, reacted with the Ehrlich reagent at room temperature and gave about 2.8 times as much color, under the conditions shown in Table III, as an equimolar quantity of UDP- X_{II} . In addition, the amino group at C-4 of X_{II} may be acylated in natural substances, and this would either decrease the reactivity with the Ehrlich reagent (e.g., see Table III for the reaction shown by the *N*-acetyl derivative), or might result in a negative test. Thus, a negative Ehrlich test would not be evidence for the absence of this sugar.

An alternate method for detecting X_{II} was therefore developed. In this procedure, the substance was dinitrophenylated and hydrolyzed with acid, and the hydrolyzate was subjected to electrophoresis. The DNP- X_{II} migrated more rapidly than did amino acid derivatives (Fig. 6); the positive charge resulted from liberation of the amino group at C-2, i.e., cleavage of the acetamido group by the acid treatment. This method suffers, of course, from the limitation that the amino group of X_{II} must be available for dinitrophenylation.

The colorimetric and dinitrophenylation methods were applied to the analysis of "C" polysaccharide (31) and to the Type XIV specific capsular polysaccharide. As shown in Table III, Type XIV polysaccharide gave negative results, but the highly purified "C" polysaccharide gave positive results by both methods, and the results suggest that this

substance contains a substantial quantity of X_{II} or similar substances, and that the amino group of the sugar is free to react with the fluorodinitrobenzene reagent.

Table III also shows that the aldehyde function of X_{II} was required for a positive Ehrlich's test (the sugar alcohols did not react), and that *N*-acetylation of the amino group at C-4 markedly reduced the reactivity with this reagent.

Glycolipids containing X_{II} . In our first studies on the biosynthesis of Type XIV polysaccharides (1), we noted the incorporation of considerable quantities of hexose into glycolipid from UDP-hexose, and the formation of small quantities of radioactive glycolipid from labeled UDP-*N*-acetylglucosamine. The hexose containing glycolipids have been characterized as glucosyl-diglyceride, and galactosyl-glucosyl-diglyceride (2).

In the present studies, the hexosamine-containing glycolipids were examined, and the labeled material was found to be X_{II} and not glucosamine or galactosamine. With the crude preparations presently available, the lipid acceptor is endogenous material in the enzyme fraction, and UDP-*N*-acetylglucosamine (or UDP-*N*-acetylgalactosamine) is converted to UDP- X_{II} prior to incorporation into the lipid. Preliminary experiments showed that UDP- X_{II} was at least as efficient a glycosyl donor as UDP-*N*-acetylglucosamine.

Two chromatographically pure ^{14}C -lipids were isolated from the incubation mixtures; the reaction mixtures were those used for the preparation of UDP- X_{II} described above. The lipids were purified by silicic acid column chromatography followed by thin-layer chromatography. While the complete structures of these lipids are not yet known, the following information has been obtained. (a) The lipids gave negative tests with the anthrone and Elson-Morgan (after acid hydrolysis) reagents, and therefore do not contain detectable quantities of hexose or hexosamine. (b) One of the lipids contained phosphorus, whereas the other did not. (c) Dinitrophenylation, followed by acid hydrolysis and paper electrophoresis, gave a product from each lipid that corresponded to the DNP-derivative of deacetylated X_{II} (see Fig. 6 C). (d) Neither the lipids nor their

saponification products gave a direct Ehrlich reaction, or a reducing sugar test. (e) Upon saponification under relatively mild conditions (2), both lipids gave water-soluble fragments containing all of the ^{14}C , and which were purified by chromatography on Dowex-50, hydrogen-form resin. Each of the lipids gave but one ^{14}C -product, and the products from the two lipids were either the same substance or were closely related. Chemical studies with the ^{14}C -water-soluble materials showed that they contained a free amino group (ninhydrin reaction, electrophoretic mobility, reaction with fluorodinitrobenzene to yield the DNP derivative described above). *N*-Acetylation of each of the ^{14}C -saponification products gave an electrophoretically neutral substance. Finally, acid hydrolysis of the saponification products gave glycerol.

The results thus far suggest that the lipids may be diglycerides where X_{II} is glycosidically bound to the glycerol moiety. The phosphorous containing lipid is, of course, more complex; perhaps it is a phosphatidyl glycerol type of molecule that contains X_{II} .

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