white powder was obtained, melting at 149.5–150.5. This product contained 10.6% phosphorus, but no nitrogen, chlorine or potassium. The infrared spectrum showed strong phosphoryl absorption and nothing else. The structure of this product is unknown.

Acknowledgment.—The authors are indebted to Caroline D. Miller for interpretation of the infrared spectra.

PAULSBORO, NEW JERSEY

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN]

The Synthesis of α-D-Glucosamine-1-phosphate and N-Acetyl-α-D-glucosamine-1-phosphate. Enzymatic Formation of Uridine Diphosphoglucosamine[1]

BY FRANK MALEY, GLADYS F. MALEY AND HENRY A. LARDY

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Procedures for the synthesis of α-D-glucosamine-1-phosphate and N-acetyl-α-D-glucosamine-1-phosphate are described. α-1-Bromo-3,4,6-tri-O-acetylglucosamine hydrobromide was treated with the triethylamine salt of diphenylphosphoric acid. Phenyl groups were cleaved from the resulting diphenyl phosphoric ester and the acetyl groups were removed by treatment with potassium methoxide to give the α-1-phosphoric ester of glucosamine as the crystalline monopotassium salt. N-Acetyl-α-D-glucosamine-1-phosphate is prepared from the dephenylated tri-O-acetyl intermediate by deacetylation under conditions which favor acyl migration from O to N. Some glucosamine-1-phosphate is formed under these conditions and may be separated from the N-acetyl compound with the aid of an ion exchange resin. The N-acetyl-α-glucosamine-1-phosphate is obtained as a crystalline monohydrate of the dipotassium salt. A new nucleotide, uridine diphosphoglucosamine, was formed when synthetic glucosamine-1-phosphate was incubated with uridine triphosphate in the presence of enzyme preparations from rat liver nuclei or from yeast.

The study of biosynthetic reactions involving glucosamine has been hampered by a lack of adequate sources of chemically pure phosphoric esters of this amino sugar. Enzymatically synthesized glucosamine-6-phosphate has been used to establish the enzymatic conversion of the -6-ester to the -1-ester,[2] and the enzymatic acetylation to produce N-acetylglucosamine-6-phosphate.[3] The latter has been shown[4] to be converted to the -1-ester by a specific mutase enzyme. The 1-ester in turn reacts enzymatically with uridine triphosphate to produce uridine diphosphoacetylglucosamine (UDPAG) and inorganic pyrophosphate. Preliminary results indicate that UDPAG may be involved in mucopolysaccharide synthesis[5] but more work, with intermediates of known purity, must be done to establish the pathways involved in the synthesis of this important class of compounds. It is in this area that abundant supplies of synthetic intermediates may be most helpful.

In a previous paper we described the chemical synthesis of D-glucosamine-6-phosphate and N-acetyl-D-glucosamine-6-phosphate.[6] It is the purpose of this paper to describe the chemical synthesis of two other biologically active intermediates, namely, α-D-glucosamine-1-phosphate and N-acetyl-α-D-glucosamine-1-phosphate.

The method of synthesis is indicated in Fig. 1. α-1-Bromo-3,4,6-tri-O-acetylglucosamine hydrobromide[7] was treated with the triethylamine salt of diphenylphosphoric acid. The phosphorylated product, II, was obtained as the crystalline hydrochloride salt. That the reaction yields products of α-configuration was conclusively established by facts to be presented below. After removal of the phenyl groups from II by catalytic hydrogenation, the acetyl groups were cleaved with potassium methoxide and the product was recrystallized as the pure monopotassium salt. It is presumably a zwitterion as depicted in Fig. 1.

N-Acetyl-α-glucosamine-1-phosphate was prepared from II by deacetylation under conditions which favor migration of acetyl groups from O to N. White[8] has shown that N-acetylglucosamine can be formed from tetra-O-acetylglucosamine in ammoniacal methanol. It has been logically assumed[9] that the acetyl group migrates from the 1- to the 2-position. In the present case, the migrating group must come from the 3-, 4- or 6-position and models of the compound in the most probable conformation demonstrate that the acetyl group on carbon atom 3 is in closest juxtaposition with the nitrogen at position 2.

The product obtained by treatment of III with ammoniacal methanol is a mixture of N-acetylglucosamine-1-phosphate and glucosamine-1-phosphate as indicated by paper chromatography, rate of acid hydrolysis and by enzymatic studies. In initial experiments the latter amounted to about 40% of the product. Conducting the hydrogenation at 0° and treating the product with anhydrous ammonia immediately thereafter did not diminish the extent of complete deacetylation. The amount of glucosamine-1-phosphate formed could be reduced to about 20% by the use of greater volumes of methanol as solvent. It is necessary to separate the two phosphoric esters since the ease of crystallization and the purity of the N-acetylglucosamine-1-phosphate are affected by the presence of glucosamine-1-phosphate. Separation of the mixture was accomplished easily with the aid of Dowex-1

(1) This paper is No. VII in the series "Phosphoric Esters of Biological Importance." Supported in part by grants from the University Research Committee and from the National Institute of Arthritis and Metabolic Diseases.

(2) D. H. Brown, *J. Biol. Chem.*, **204**, 877 (1953).

(3) D. H. Brown, *Biochim. et Biophys. Acta*, **16**, 429 (1955).

(4) L. F. Leloir and C. E. Cardini, *ibid.*, **12**, 15 (1953).

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(7) J. C. Irvine, D. McNicoll and A. Hynd, *J. Chem. Soc.*, **99**, 250 (1911).

(8) T. White, *ibid.*, 1498 (1938).

(9) A. B. Foster and M. Stacey, *Adv. in Carbohydrate Chem.*, **7**, 247 (1952).

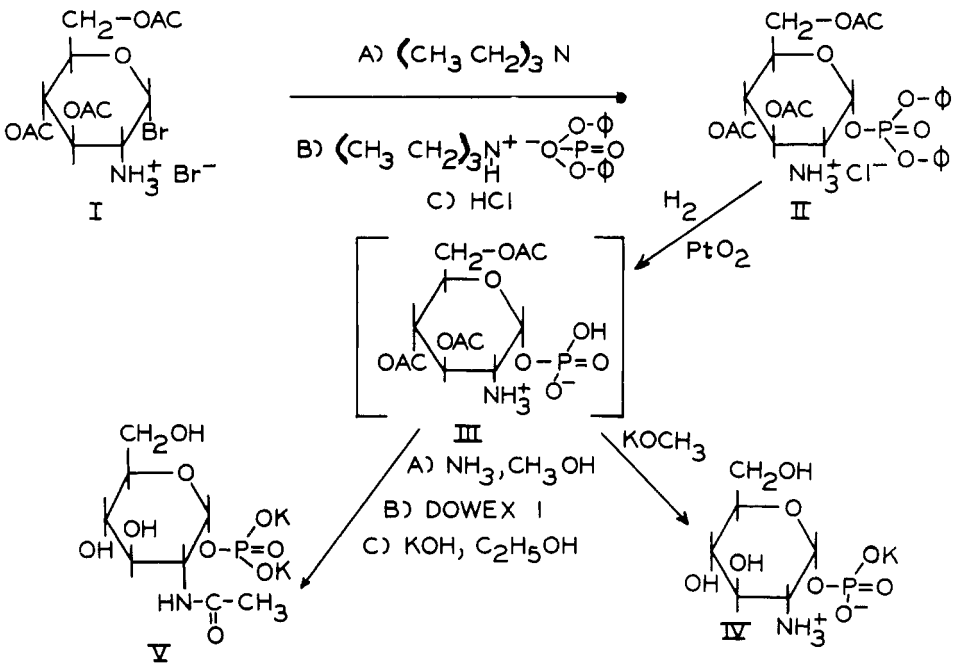

Fig. 1.—Scheme for synthesis of α-D-glucosamine-1-phosphate and N-acetyl-α-D-glucosamine-1-phosphate.

anion exchange resin as described in the Experimental section. The N-acetyl compound is then crystallized as the monohydrate of the dipotassium salt.

The configuration of these two phosphorylated glucosamines is established readily by an application of Hudson's isorotation rules.[10] The pertinent data are shown in Table I together with a predicted rotation for the, as yet, unknown β isomer. The 2B values for D-glucosamine and for methyl-D-glucosaminide are 25,860 and 23,638. Since the molar rotation of the synthetic glucosamine-1-phosphate is +29,730 the predicted molar rotation of the unknown isomer is approximately −4000 to −6000. The synthetic compound is, therefore, clearly of α-configuration. Since the glucosamine-1-phosphate and N-acetylglucosamine-1-phosphate are derived from a common precursor, the N-acetylphosphate ester must also be of α-configuration. This conclusion is confirmed by the independent calculation shown in the lower part of Table I.

Among other physical constants which were determined, the apparent pK values deserve comment. The dissociation constant for the second acid group of glucosamine-1-phosphoric acid was found to be 5.4 and that of N-acetylglucosamine-1-phosphoric acid was 6.1. In the former, the adjacent free amino group greatly enhances the acidity of the phosphoric acid.

As was first observed by Brown, glucosamine-1-phosphate is considerably more resistant to acid hydrolysis than are the common aldose-1-phosphates. The greater stability of methylglucosaminides than of methyl glucosides has been ascribed by Moggridge and Neuberger[11] to the proximity of the positive charge on the amino group. This explanation might also apply to the case of the hexosamine-1-phosphates.

During the course of this investigation Leloir and Cardini[12] published a procedure for the preparation of N-acetylglucosamine-1-phosphate of about 84% purity by treating α-chloro-tetraacetylglucosamine with trisilver phosphate. They too concluded that their compound is of α-configuration and the calculated molecular rotation of the calcium salt was higher than that reported here for the pure dipotassium salt. We have attempted, unsuccessfully, to treat triethylamine diphenyl phosphate with the α-chloro-tetraacetylglucosamine; the N-acetyl group apparently inhibits the reaction since only small amounts of triethylamine hydrochloride were recovered.

Experimental

Materials and Methods.—Inorganic phosphorus was determined by the method of Fiske and SubbaRow,[13a] total phosphate by that of King.[13b] N-Acetylglucosamine was determined after hydrolysis of N-acetylglucosamine-1-phosphate in 0.1 N HCl for 10 min. by the method of Reissig, *et al.*[14] This treatment results in complete hydrolysis of the phosphate without appreciable hydrolysis of the acetyl group. Glucosamine was measured by the method of Morgan and Rondle.[15] Nucleotides were separated by a procedure similar to that described by Hurlbert, *et al.*[16] All melting points were determined with Anschütz thermometers calibrated by the National Bureau of Standards.

1-Diphenylphosphoro-3,4,6-tri-O-acetyl-α-D-glucosamine Hydrochloride (II).—A suspension of 5.31 g. of α-1-bromo-3,4,6-tri-O-acetylglucosamine hydrobromide (I)[7,17] in 80

(10) "Collected Papers of C. S. Hudson," Vol. I, Academic Press, Inc., New York, N. Y., 1946.

(11) R. C. G. Moggridge and A. Neuberger, *J. Chem. Soc.*, 745 (1938).

(12) L. Leloir and C. E. Cardini, *Biochim. et Biophys. Acta*, **20**, 33 (1956).

(13) (a) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925); (b) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(14) J. L. Reissig, J. L. Strominger and L. F. Leloir, *J. Biol. Chem.*, **217**, 959 (1955).

(15) C. J. M. Rondle and W. T. J. Morgan, *Biochem. J.*, **61**, 586 (1955).

(16) R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *J. Biol. Chem.*, **209**, 23 (1954).

(17) To obtain this compound in a pure, colorless condition it was found necessary to treat chloroform solutions of it with large amounts of Norite A.

TABLE I

ESTABLISHMENT OF α-CONFIGURATION FOR GLUCOSAMINE-1-PHOSPHATE AND N-ACETYLGLUCOSAMINE-1-PHOSPHATE

All rotations were measured in water. The data for the non-phosphorylated compounds are from Foster and Stacey.[9] Calculated approximate values for the rotation of the β isomers of glucosamine-1-phosphate and N-acetylglucosamine-1-phosphate are shown in brackets.

Compound	α-Isomers		β-Isomers			
	$[\alpha]_D$	$[M]_D$	$[\alpha]_D$	$[M]_D$	2A	2B
D-Glucosamine hydrochloride	+100°	+21,550	+20°	+ 4,310		25,860
Methyl-D-glucosaminide HCl	+127	+29,146	−24	− 5,508	34,654	23,638
D-Glucosamine-1-phosphate	+100	+29,730	[−20	− 6,000]		
Methyl-N-acetyl-D-glucosaminide	+105	+24,675	−43	−10,105	34,780	14,570
N-Acetyl-D-glucosamine-1-phosphate	+ 79	+29,800	[−40	−15,000]		

ml. of dry benzene was treated with 1.6 ml. of triethylamine; this resulted in the immediate precipitation of triethylamine hydrobromide. A solution of 2.75 g. of diphenylphosphoric acid[18] in 20 ml. of benzene containing 1.6 ml. of triethylamine was then added and the suspension was shaken for one hour. After about four hours, the solution was filtered to remove the triethylamine hydrobromide and the filtrate was concentrated *in vacuo* to a thin sirup. Forty ml. of 2 *N* HCl was added slowly to the concentrate while shaking to precipitate the product. After storing at 5° overnight, the precipitate was collected on a filter and washed with ether. If dried *in vacuo* over P_2O_5 and soda-lime at this stage the yield was 5.23 g. Best yields were about 80%. Recrystallization was accomplished by adding ether to a methanol solution of the product. The crystalline hydrochloride decomposed at 137–138°, $[\alpha]^{23}D$ +110° (*c* 2.44, CH_3OH).

Anal.[19] Calcd. for $C_{24}H_{29}O_{11}NPCl$ (573.9): C, 50.22; H, 5.09; N, 2.44; P, 5.40. Found: C, 50.45; H, 5.40; N, 2.12; P, 5.13.

α-D-Glucosamine 1-Monopotassium Phosphate (IV).—A solution of 1.21 g. of II in 30 ml. of absolute methanol was hydrogenated with 100 mg. of PtO_2 as catalyst at room temperature and atmospheric pressure. About 2.5 hr. were required for eight mole equivalents of hydrogen to be consumed. The acidic solution was filtered, cooled to 0–5° and 4.5 ml. of 1.24 *N* potassium methoxide was added. After about 24 hr. at this temperature crystals in the form of rosettes began to appear; 3–4 days were allowed for the product to accumulate. The yield was 0.365 g.; yields varied from 50 to 60%. The product did not give a positive Fehling test. Analyses indicated that the obtained product was a mixture of mono- and dipotassium salts. The pure monopotassium salt could be obtained by dissolving the crude salt in water (0.1 g. per ml.), adding about 10 volumes of methanol and placing at 0–5° until crystallization was complete.

The sample was dried to constant weight at 100° prior to analysis, $[\alpha]^{23}D$ +100° (*c* 1.98, H_2O). The hydrolysis rate constant at 100° in 1 *N* HCl was 0.23 $min.^{-1}$. In 0.15 *M* KCl, pK_2' = 5.4, pK_3' = 8.5; in H_2O, pK_2' = 5.5, pK_3' = 8.7.

Anal. Calcd. for $C_6H_{13}O_8NPK$ (297.3): C, 24.24; H, 4.41; N, 4.71; P, 10.42. Found: C, 24.06; H, 4.54; N, 4.79; P, 10.34.

Analysis of another preparation demonstrated that the ratio of P to K was 1.

N-Acetyl-α-D-glucosamine Dipotassium 1-Phosphate (V).—A solution of 1.013 g. of II in 85 ml. of absolute methanol was hydrogenated with 150 mg. of PtO_2 as catalyst. After eight mole equivalents of hydrogen were taken up, the acidic solution was filtered and cooled to 0–5°. Anhydrous ammonia was bubbled into the solution for 5 min.; the ammoniated solution was allowed to stand at room temperature for 2 hr. and then placed at 5° overnight. The small amount of precipitate that formed was separated by filtration and discarded. About 100 ml. of methanol was added to the filtrate which was then concentrated under reduced pressure to remove the ammonia. The concentrated solution was brought to about 100 ml. with methanol, an equal volume of ether was added and the precipitate that formed was allowed to settle at 5° for a few hours. The precipitate was then collected by filtration and dried; yield 0.434 g. This product is a mixture of the ammonium salts of glucosamine-1-phosphate and N-acetylglucosamine-1-phosphate; the latter usually represented 75–80% of the total phosphate.

Chromatographic Separation.—The only limiting factor involved was found to be column size. The following example represents a typical separation, which can be modified easily to suit the experimenter's needs. A 3-ml. solution containing 110 mg. of the crude mixture was brought to *p*H 8.5 with 2 *N* NH_4OH and placed on a 14 × 2.5 cm. column of Dowex 1-X10, 200–400 mesh in the Cl form. Thorough washing of the resin with 4 *N* HCl and 4 *N* NaOH alternately, prior to use, is recommended. About 5 ml. of water was added to wash in the material. The column was eluted with 200 ml. of 0.005 *N* HCl and the eluate was collected in 10-ml. fractions. Analysis of 1-ml. aliquots for acid-labile phosphorus revealed a small amount (about 1% of the total) in tubes 2–3 and a larger amount in tubes 8–15. After tube 20, 200 ml. of 0.05 *N* HCl was used as the eluting agent, and 0.2 ml. was taken from each 10-ml. fraction for phosphate analysis. A peak appeared from tubes 25–33. Analysis for N-acetylglucosamine after acid hydrolysis revealed its presence in tubes 2–3 and 25–33. The first fraction was most probably a small amount of N-acetylglucosamine-1-phosphate that was not held by the column; the second fraction (8–15) was glucosamine-1-phosphate. The fraction from 25–33 was brought to *p*H 10–11 with 2 *N* KOH and concentrated to 3–5 ml. under reduced pressure. About 10 volumes of 95% ethanol were added and the turbid solution placed at 0–5°. The turbidity is due to an oil which usually crystallized in the form of needles on standing in the cold for 24 hr. Seeding of the turbid solution with crystalline potassium N-acetylglucosamine-1-phosphate ensured crystallization in all cases.[20] The yield in this case was 70 mg. Recrystallization resulted in high recoveries (*ca.* 80%) of pure compound. Neither the impure nor the pure product gave a positive Fehling test.

This compound, like glucosamine-1-phosphate, is very hygroscopic, but it was dried *in vacuo* without heating prior to analysis. Due to the difficulty in obtaining accurate weighings, total phosphate was used to determine concentrations for specific rotation determinations, based on the anhydrous molecular weights of the compounds; $[\alpha]^{23}D$ +79° (*c* 0.954, H_2O). The hydrolysis rate constant at 37° in 1 *N* H_2SO_4 was found to be 3.7×10^{-3} $min.^{-1}$ which is similar to that reported by Leloir and Cardini,[12] 4.1×10^{-3} $min.^{-1}$ (their recorded value × 2.3). In H_2O $pK_1' < 1.5$; $pK_2' = 6.1$.

Anal. Calcd. for $C_8H_{14}O_9NPK \cdot H_2O$ (395.4): C, 24.30; H, 4.08; N, 3.54; P, 7.8. Found: C, 24.38; H, 4.34; N, 3.57; P, 7.7.

After hydrolysis of the compound in 0.1 *N* HCl for 10 minutes at 100°, the ratio of free N-acetyl glucosamine to inorganic phosphate was found to be 1.

Biological Activity

Brown[2] has described the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate by phosphoglucomutase. However, the latter sugar

(18) Generously contributed by the Dow Chemical Co., Midland, Mich. The compound contained two moles of water which were removed by drying *in vacuo* over P_2O_5 and it was then recrystallized from chloroform by the addition of petroleum ether (Skelly B).

(19) C, H and N analyses were performed by Micro-tech Laboratories Skokie, Ill.

(20) It is advised to seed with crystalline material which does not give a precipitate with $AgNO_3$. Trace amounts of KCl in the seed crystals encourage the co-crystallization of KCl with the desired compound.

was not isolated or characterized. The identity of the chemically prepared glucosamine-1-phosphate has been established by assay with crystalline phosphoglucomutase. We are indebted to Dr. S. Roseman for these studies. He found that 78% of the synthetic glucosamine-1-phosphate was converted to the -6-phosphate at equilibrium. This agrees with the equilibrium obtained by Brown[2] using glucosamine-6-phosphate.

Since it was shown by Smith and Mills[21] that uridine diphosphoglucose (UDPG) can be pyrophosphorylated in the presence of a nuclear extract to form uridine triphosphate (UTP) and glucose-1-phosphate, it was of interest to determine whether glucosamine-1-phosphate would undergo the reversal of this reaction. As can be seen in Fig. 2B, it did. In the presence of glucosamine-1-

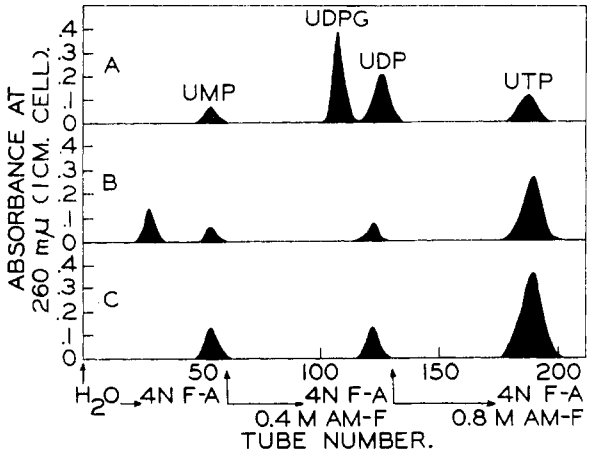

Fig. 2.—Synthesis of uridine diphosphoglucosamine by an extract of rat liver nuclei. The reaction mixtures contained 3 μmoles of UTP, 15 μmoles of $MgSO_4$, 150 μmoles of tris-hydroxymethylaminomethane *p*H 8.0, 30 μmoles of KF and 1.0 ml. of nuclei extract prepared according to the procedure of Mills, *et al.*[22] Substrate additions were: A, 10 μmoles of glucose-1-phosphate; B, 25 μmoles of glucosamine-1-phosphate; C, none. The final volume was 4 ml. After incubation at 37° for 30 minutes the reaction was stopped by addition of 2 ml. of cold 10% trichloroacetic acid. After centrifugation the supernatant solution was neutralized and chromatographed on a Dowex-1 formate column according to the gradient elution technique of Hurlbert, *et al.*[16] Fractions of 5 ml. were collected. The first peak in section B is uridine diphosphoglucosamine.

phosphate, UTP and a rat liver nuclear extract, a new, fast-moving glucosamine-containing nucleotide appeared. The nucleotide is believed to be uridine diphosphoglucosamine by analogy to the compound formed from glucose-1-phosphate and UTP in the uridyl transferase reaction.[21,23] Evidence for this was obtained by analysis of the chromatographically isolated nucleotide. Uridine, acid labile phosphorus, total phosphorus and glucosamine were found to be present in the ratio 1.0:1.2:1.9:1.0, respectively. It has been found that an extract of yeast will also convert glucosamine-1-phosphate to uridine diphosphoglucosamine in the presence of UTP. Whether the *in vitro* formation of this glucosamine-containing nucleotide is of biological significance is not yet known but its possible involvement in galactosamine synthesis *via* a galactowaldenase-type reaction or in mucopolysaccharide biosynthesis cannot be neglected.

The corresponding nucleotide containing N-acetylglucosamine (UDPAG) was discovered in yeast[24] and occurs also in liver.[21,25] Since it is formed from UTP and N-acetylglucosamine-1-phosphate by yeast extracts[5] and cleaved to UTP and presumably N-acetylglucosamine-1-phosphate by an extract of liver nuclei in the presence of pyrophosphate,[21] it would be expected that the nuclear extract could also effect its synthesis. However, no detectable synthesis of UDPAG occurred when the chemically prepared N-acetylglucosamine-1-phosphate was incubated with an extract of rat liver nuclei in the presence of UTP. The authenticity of the synthetic N-acetylglucosamine-1-phosphate was affirmed, however, by the finding that yeast extract converts it to UDPAG (Fig. 3). It was also found that synthetic N-

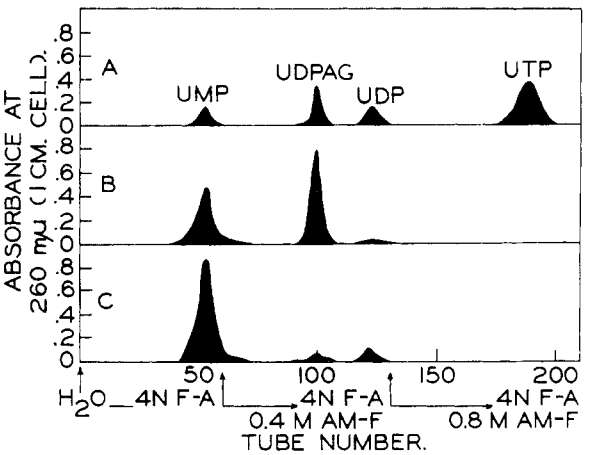

Fig. 3.—Enzymatic synthesis of uridine diphosphoacetylglucosamine from chemically prepared N-acetylglucosamine-1-phosphate: A, chromatogram of an authentic sample of UDPAG added to reaction mixture C of Fig. 2 after deproteinization with trichloroacetic acid. B and C, the reaction mixtures contained 5 μmoles of UTP, 15 μmoles of $MgSO_4$, 130 μmoles of tris-hydroxymethylaminomethane *p*H 8.0, 60 μmoles of KF and 2.0 ml. of dialyzed yeast extract prepared according to the procedure of Jones, *et al.*[26] Substrate additions were: B, 15 μmoles of N-acetylglucosamine-1-phosphate; C, none. The final volume was 4.0 ml. and the reaction mixtures were incubated at 30° for 60 minutes. Thereafter the procedure was the same as in Fig. 2.

acetylglucosamine-6-phosphate[6] can be converted to this nucleotide but to a lesser extent than the

(21) E. E. B. Smith and G. T. Mills, *Biochim. et Biophys. Acta*, **13**, 386 (1954).

(22) G. T. Mills, R. Ondarza and E. E. B. Smith, *Biochim. et Biophys. Acta*, **14**, 159 (1954).

(23) A. Munch-Peterson, H. M. Kalckar, E. Cutolo and E. E. B. Smith, *Nature*, **172**, 1036 (1953).

(24) E. Cabib, L. Leloir and C. E. Cardini, *J. Biol. Chem.*, **203**, 1055 (1953).

(25) R. B. Hurlbert and V. R. Potter, *ibid.*, **209**, 1 (1954).

(26) M. E. Jones, S. Black, R. M. Flynn and F. Lipman, *Biochim. et Biophys. Acta*, **12**, 141 (1953).

corresponding -1-phosphate ester. This can be explained by the unfavorable equilibrium[27] between the 1- and 6-phosphates and the removal of N-acetylglucosamine-6-phosphate from the reaction mixture by enzymes which convert it to other hexose phosphates.[11]

(27) J. L. Reissig, *J. Biol. Chem.*, **219**, 753 (1956).

MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, EMORY UNIVERSITY]

The Formation of Pyridoxal and Pyridoxal 5-Phosphate Hydrazones[1]

BY RONALD G. WIEGAND

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Specific reaction rates for the formation of pyridoxal 5-phosphate hydrazones from pyridoxal 5-phosphate and a series of hydrazides have been determined, as well as the experimental activation energies for the reactions. The acid dissociation constants of the hydrazides are shown to be linearly related to the specific reaction rates. Data for the reaction of hydrazides with pyridoxal are also given.

Hydrazides have been shown to inhibit several enzyme systems dependent on pyridoxal 5-phosphate (PLP) as a coenzyme.[2-4] The mechanism of this inhibition has been postulated to be the formation of a hydrazone with pyridoxal[5,6] or pyridoxal 5-phosphate.[3]

Vilter and co-workers[5] have suggested that the increased excretion of vitamin B_6 activity in humans following chronic treatment with isonicotinyl hydrazide (INH) is due to excretion of pyridoxal isonicotinyl hydrazone. They further suggest that this complex is formed directly from the interaction of INH and pyridoxal. However, these authors present no evidence to support their suggestion. Williams and Abdulian[6] demonstrated pyridoxal semicarbazone in the urine of semicarbazide treated dogs, which is the only instance, to the author's knowledge, that a pyridoxal hydrazone has been demonstrated to be formed *in vivo*. They conclude that convulsant hydrazides, including INH, combine with pyridoxal to form the corresponding pyridoxal hydrazone.

Davison[3] suggests that INH combines with the phosphorylated form of pyridoxal, and presents experimental activation energies for the reaction of INH with PLP in the presence and absence of B_6-requiring enzymes (16 and 14 kcal./mole, respectively). This similarity of energies of activation is considered by Davison as evidence supporting the initial interaction of INH with pyridoxal phosphate, forming the phosphorylated hydrazone.

The present study was undertaken to clarify the reaction between a series of convulsant hydrazides and both pyridoxal and pyridoxal 5-phosphate. Any correlation between the physicochemical characteristics of the reactions and the convulsant activity of the hydrazides[7] would be of interest. The relative rates of reaction with both forms of vitamin B_6, as well as the experimental activation energies of the reactions, would be of value in defining more precisely the mechanism of the inhibition of enzymatic reactions by hydrazides.

Experimental

Dissociation Constants of Hydrazides.—The acid dissociation constants of thiosemicarbazide (TSC), thiocarbohydrazide (TCH), semicarbazide (SC) and carbohydrazide (CH) were determined at 25.0 ± 0.3° by titration with 0.0984 *N* HCl. A Beckman model G *p*H meter was used to measure *p*H. Two and a half millimoles of hydrazide was dissolved in 25 ml. of redistilled water for titration, except in the case of thiocarbohydrazide where 50 ml. of water was used to allow solution. pK_a values were taken as the points of half neutralization. Without corrections for ionic strength, the pK_a values obtained are reported in Table I. The pK_a of semicarbazide determined under these conditions agrees well with the value of 3.68 reported by Bartlett.[8]

Specific Reaction Rates for Formation of Hydrazones of Pyridoxal and Pyridoxal 5-Phosphate.—All reactions were run in 0.05 *M* phosphate buffer, *p*H 7.4, under conditions of constant total acidity.[9] The reaction was followed spectrophotometrically[3] in a Beckman model DU spectrophotometer equipped with thermospacers for temperature regulation of the cell compartment. The compartment temperature was constant within 0.5°. The increase in optical density of the solutions was measured at a wave length chosen to give a large difference in molar extinction coefficient between pyridoxal or pyridoxal-5-phosphate (PLP) and the corresponding hydrazone. The wave lengths used were 288 mμ for the formation of PLP semicarbazone (PLPSC) and pyridoxal and PLP carbohydrazones (PLCH and PLPCH), 306 mμ for PLP thiocarbohydrazone (PLPTCH), 315 mμ for PLP thiosemicarbazone (PLPTSC), and 330 mμ for the formation of PLP isonicotinyl hydrazone (PLPINH). The ultraviolet absorption spectra of free PLP and the PLP hydrazones are given in Fig. 1. The spectra were obtained using a Beckman model DK-2 ratio recording spectrophotometer.

The second-order reaction for the formation of pyridoxal or PLP hydrazones was made to conform to first-order kinetics by use of a 200-fold molar excess of hydrazide. Two ml. of 0.10 m*M* pyridoxal (Nutr. Biochem. Corp.) or PLP (Calif. Found. for Biochem. Res., 100 ± 3% pure) was mixed with 2.0 ml. of 20 m*M* hydrazide at the start of the reaction, both solutions having been brought to temperature equilibrium before mixing. Optical density readings were taken at 30 second to 5 minute intervals, depending on the speed of the reaction, until the reaction was about three-quarters complete. Readings were then taken until constant. A 10 m*M* hydrazide blank in 0.05 *M* phosphate buffer was used. Optical density readings for 0.05 m*M* pyridoxal or PLP in 0.05 *M* phosphate buffer (OD_0) were taken at the wave lengths used for the rate de-

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