THE UNIVERSITY OF MICHIGAN
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ASPECTS OF HEXOSAMINE METABOLISM

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<td>AcCoA</td>
<td>acetyl coenzyme A</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>DPN⁺</td>
<td>diphosphopyridine nucleotide</td>
</tr>
<tr>
<td>DPNH</td>
<td>reduced diphosphopyridine nucleotide</td>
</tr>
<tr>
<td>Galm</td>
<td>galactosamine = 2-amino-2-deoxy-D-galactose</td>
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<td>Galm-1-P</td>
<td>galactosamine 1-phosphate</td>
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<td>Galm-6-P</td>
<td>galactosamine 6-phosphate</td>
</tr>
<tr>
<td>G-6-P</td>
<td>glucose 6-phosphate</td>
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<tr>
<td>Gm</td>
<td>glucosamine = 2-amino-2-deoxy-D-glucose</td>
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<td>Gm-6-P</td>
<td>glucosamine 6-phosphate</td>
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<td>Gmic H⁺</td>
<td>D-glucosaminic acid</td>
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<td>GSH</td>
<td>glutathione</td>
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<td>KDG</td>
<td>2-keto-3-deoxy-D-gluconic acid</td>
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<td>KDPG</td>
<td>2-keto-3-deoxy-6-phospho-D-gluconic acid</td>
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<td>ME</td>
<td>2-mercaptoethanol</td>
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<td>FEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal phosphate</td>
</tr>
<tr>
<td>UDPAGalm</td>
<td>uridine diphosphoacetylgalactosamine</td>
</tr>
<tr>
<td>UDPAGm</td>
<td>uridine diphosphoacetylglucosamine</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
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GENERAL INTRODUCTION
INTRODUCTION

The hexosamines are widely distributed in nature, generally as integral components of the high molecular weight mucoid substances. They are found as polymers of the individual hexosamines, e.g., chitin (N-acetyl-D-glucosamine), or in combination with other carbohydrate components as in hyaluronic acid (N-acetyl-D-glucosamine and D-glucuronic acid), and chondroitin sulfuric acid (N-acetyl-D-galactosamine, D-glucuronic or L-iduronic acid, and sulfuric acid).

Definitive characterization of the hexosamines is a significant problem to investigators in this area. Part I of this thesis is concerned with the characterization of D-galactosamine by a method which adequately distinguishes this compound from D-glucosamine.

Part II of this thesis is concerned with some aspects of D-galactosamine metabolism. D-Galactosamine 6-phosphoric and N-acetyl-D-galactosamine 6-phosphoric acids were chemically synthesized, and the enzymatic acetylation of D-galactosamine 6-phosphate by enzyme preparations from fungal and mammalian tissues was studied.

Part III of the thesis is a study of the metabolism of D-glucosaminic acid in bacteria. Although it has been indicated that D-glucosaminic acid is synthesized from D-glucosamine by certain bacteria, no information is available concerning the further metabolism of this compound.

Figure 1 indicates the structural formulae of the substances which are primarily involved in the investigations in this thesis.
2-Amino-2-deoxy-D-glucose (Glucosamine)
2-Amino-2-deoxy-D-galactose (Galactosamine)

2-Acetamido-2-deoxy-D-glucose (N-acetylglucosamine)
2-Acetamido-2-deoxy-D-galactose (N-acetylglactosamine)

2-Amino-2-deoxy-D-gluconic acid (Glucosaminic acid)

Figure 1. Structural Formulae of the Hexosamines and Related Compounds.
PART I

PREPARATION OF N-CARBOBENZYLOXY-D-GALACTOSAMINE
A. HISTORICAL REVIEW

1. Discovery of D-Galactosamine

Although the amino sugars are now known to be widely distributed in nature, the early isolation and characterization of these sugars proved a difficult and confusing problem. The first amino sugar isolated was D-glucosamine, and was obtained by Ledderhose (1) in 1878 by acid hydrolysis of lobster shells. A period of thirty-six years elapsed before it was recognized by Levene and La Forge (2) that a hexosamine other than Gm was a component of chondroitin sulfuric acid. In his monograph on the hexosamines (3), Levene discusses the historical development of this discovery. Chondroitin sulfuric acid was first isolated from cartilage by Fischer and Boedeker in 1854 (4) and subsequently purified by Krukenberg in 1884 (5) and Mörner in 1889 (6). In 1891 Schmiedeberg (7) recognized the presence of uronic acid and hexosamine (which he believed to be Gm) in chondroitin sulfuric acid. However, later workers differed with Schmiedeberg's conclusions. Thus, Orgler and Neuberg (8) concluded that chondroitin sulfuric acid did not contain hexosamine, but a nitrogenous component - aminotetrahydroxy caproic acid, a hexose, and no uronic acid. Frankel (9) suggested that the nitrogenous component was an aminoglucuronic acid, while Sawjalow (10) reported the presence of 2,5-anhydrohexose and a base of the composition of $C_7\text{H}_9\text{O}_7\text{N}$. Levene and La Forge in 1913 (11), presented evidence that Schmiedeberg's original postulations were essentially correct - that chondroitin sulfuric acid contained a uronic acid, which they identified as glucuronic acid and a hexosamine which they believed to be Gm. However, in 1914 (2)
these authors realized that the hexosamine was not Gm, although it exhibited the same elementary composition. Gm and chondrosamine (the name designated by Levene and La Forge for the unknown hexosamine) differed in their physical properties, e.g., the hydrochlorides of the two sugars differed in crystal form, solubility in water, decomposition points and specific rotations; the phenyllosazone derivatives differed in melting points and specific rotations. Levene and La Forge's identification of chondrosamine was based on the following experimental evidence (12): (a) chondrosamine yielded a phenyllosazone identical with that prepared from D-galactose, which therefore indicated the same spacial configuration of the hydroxyl groups around C-3, 4, 5, 6; (b) confirmation of these findings was obtained by the synthesis of chondrosamine from D-lyxose. Condensation of D-lyxose with ammonia yielded D-lyxosimine; addition of HCN to the imino compound, followed by hydrolysis, gave the epimeric 2-amino-2-deoxy-D-hexonic acids. Conversion of the acids to the lactones and subsequent reduction yielded the epimers, 2-amino-D-talose and 2-amino-D-galactose, one of which was identical with chondrosamine. Levene and La Forge, however, did not differentiate between the two epimers and the problem of the configuration of the amino group on C-2 remained undecided and a matter of speculation.

In 1946, the identity of chondrosamine as 2-amino-2-deoxy-D-galactose was unequivocally established by James et al. (13). The action of ammonia on 1,6:2,3-dianhydro-β-D-talose (I) gave a small amount of
the D-idose derivative (II) with a major portion of the D-galactose derivative (III). (III) when treated with HCl gave 2-amino-D-galactose hydrochloride (IV). This was identical with naturally occurring chondrosamine in optical rotation, X-ray powder diffraction patterns, and also yielded derivatives exhibiting the same properties.
2. Distribution of Galactosamine (Galm)

Galm exists in natural substances as a component of higher molecular weight compounds and usually as its N-acetyl derivative. While it was first discovered in chondroitin sulfuric acid of cartilage by Levene and La Forge (2), it is now known to have a much wider distribution. It may be noted that the chondroitin sulfuric acids still remain a major source of galactosamine.

Investigations carried out chiefly by Meyer et al. (14,15) have demonstrated that the sulfated mucopolysaccharides from connective tissues are more numerous and more complex than had previously been believed. The sulfated mucopolysaccharides containing Galm are now known to be composed of at least three different fractions, namely, chondroitin sulfuric acids A, B and C. Both chondroitin sulfuric acid A and C contain equimolar amounts of Galm, acetic acid, D-glucuronic acid and sulfuric acid; both are hydrolyzed by testicular hyaluronidase and resistant to bacterial hyaluronidase. They differ from each other in the solubilities of their calcium salts and in their optical rotations.

Chondroitin sulfuric acid A has been found in hyaline cartilage, bovine cornea, bone, bovine nasal septum, rib cartilage of newborn babies, ox ligamentum nuchae and in bovine aorta. Chondroitin sulfuric acid C appears as a minor constituent of cartilage and bone, of tendon, heart valves, umbilical cord and the culture fluids from tissue cultures of fibroblasts from bone, human skin and rat subcutaneum. Chondroitin sulfuric acid B appears to be identical with β-heparin isolated by Marbet and Winterstein (16) and was distinguished from A and C by its optical
rotation, solubility of its calcium salt, and resistance to testicular hyaluronidase. It contains equimolar amounts of Galm, acetic acid, sulfuric acid and uronic acid, but in this case L-iduronic acid is the major uronic acid component (15,17). Chondroitin sulfuric acid B has been shown to occur in pig skin, tendon, heart valves, aorta and ligamentum nuchae (14). Chondroitin, identified as an analogue of chondroitin sulfuric acid A or C (18) has been isolated free of or very low in sulfate from bovine cornea.

Other sources of Galm are the blood group substances (19); glycolipids of stroma of erythrocytes (20-23); gangliosides (24,25); submaxillary mucin (24), and other muco substances (26-29); acid mucopolysaccharides of serum (30); thymus tissue (31); human milk (32) and soil (33). Galm has been reported to be a component of bacterial cell walls (34,35); bacterial polysaccharides (36-38), and has been found as a partially acetylated polymer in the culture fluid of Aspergillus parasiticus, and is possibly present as a fully acetylated polymer (analogous to chitin) in the mycelia (39).

3. Other Hexosamines Found in Nature

mannosamine - (2-amino-2-deoxy-D-mannose), obtained as a product of enzymatic degradation of sialic acid (40).
gulosamine - (2-amino-2-deoxy-D-glucose), isolated from streptothricin and streptolin B (41).
muramic acid - (3-O-α-carboxyethylhexosamine), isolated from spores and bacterial cell walls (42).
N-methyl-L-glucosamine, isolated from streptomycin (43).
6-amino-6-deoxy-D-glucose, isolated from kanamycin (44).
kanosamine - (3-amino-3-deoxy-D-glucose), isolated from kanamycin (45).
fucosamine - (2-amino-2-deoxy-fucose), isolated from a bacterial polysaccharide (46).

desosamine - (3-dimethylamino-3,4,6-deoxy-hexose), isolated from erythromycin (47).

mycaminose - (3-dimethylamino-3,6-deoxy-hexose), isolated from magnamycin (48).

talosamine - (2-amino-2-deoxy-D-talose), isolated from cartilage (49,50). Whether this sugar is naturally occurring or is formed from Galm during the isolation procedures is not known.

4. Characterization of Hexosamines

a. Detection

As indicated above, the hexosamines are widely distributed in biological materials. However, their identification has been impeded by the lack of adequate methods of characterization. After liberation of the hexosamines from biological substances (usually accomplished by acid hydrolysis), colorimetric reactions are available that can be used for quantitative and qualitative measurement, which are relatively specific for the 2-amino sugars. These are the Elson-Morgan reaction or one of its modifications (51-55); and the Dische-Borenfreund method (56). The N-acyl derivatives can be measured by the Morgan-Elson procedure, or by its modifications (57,58). These methods, as originally described, however, will not differentiate between Gm, Galm, or other amino sugars present in the mixture. Modifications of the above methods have been applied for the differentiation and analysis of mixtures of Gm and Galm (59,60). Leskowitz and Kabat (61) analyzed mixtures containing Gm and Galm by reduction of the amino sugars to the hexosaminitolts with subsequent conversion to the dinitrophenyl derivatives, followed by separation on silica
gel and final analysis by colorimetry. The method of Gardell (62) has been widely used for the separation of Gm and Galm on Dowex-50 ion-exchange resin, followed by quantitative analysis of the eluates.

Paper chromatography for the detection of amino sugars has met with varied success (63,64). Although solvent systems are available for the separation of the amino sugars from hexoses and amino acids, the separation of the individual hexosamines from one another is more difficult. A separation of the 2,4-dinitrophenyl derivatives of Gm and Galm has been achieved in butanol-ethanol-borate (63). The N-acyl derivatives have been separated on borate treated papers (65). Particularly successful has been the method of Stoffyn and Jeanloz (66) and Gardell (67), whereby Gm and Galm are degraded by treatment with ninhydrin to the corresponding pentoses, arabinose and lyxose respectively, which can be separated by paper chromatography.

b. Characterization

The above methods, although useful for the estimation and the preliminary identification of amino sugars cannot be used as definitive evidence for their characterization. X-ray powder diffraction patterns have been successful in this area since the patterns obtained with the hydrochlorides of Gm, Galm, and mannosamine are distinctive and permit the identification of small amounts of material (68). In order to fully establish the identity of an amino sugar, it is also desirable to obtain a well-characterized crystalline derivative. Only partial success has been achieved. The following characteristics of a good derivative are noted: (a) it must be easily prepared, (b) stable and crystalline,
(c) applicable to small amounts of material, (d) its physical characteristics must be specific, e.g., melting point, (e) although not entirely necessary, it would be an advantage to regenerate the original amino sugar easily, and (f) it would be desirable to prepare a derivative which was easily separable from complex mixtures of other materials. This last point loses its importance however, since ion exchange chromatography can be successfully applied to isolate the amino sugar free from other contaminants.

Although numerous derivatives of Gm and fewer for Galm have been reported [see (63) and (64)], they have not been considered suitable for adequate characterization. The Schiff bases (69-73), have been regarded as somewhat satisfactory, particularly in the isolation of the amino sugars from complex mixtures. However their role as suitable derivatives for the final characterization of an unknown hexosamine can be criticized on the basis of their lack of stability and difficulty in purification (74).

In 1937 Chargaff and Bovarnick (75) described a procedure for the preparation of N-carbobenzyloxy-D-glucosamine which could be used for the isolation of this sugar from neutral sugars or amino acids. N-Carbobenzyloxy-D-glucosamine was stable and was obtained in essentially quantitative yield. Furthermore, glucosamine hydrochloride could be regenerated from the derivative by hydrogenolysis. The preparation of other N-carbobenzyloxy derivatives of amino sugars therefore appeared desirable. Part I of this investigation describes modifications of the original Chargaff and Bovarnick procedure for the preparation of derivatives of small amounts of Gm and Galm. The preparation of N-carbobenzyloxy-D-galactosamine has not been previously achieved, although attempts to
synthesize this derivative have been reported (72). The apparent difficulty was in the isolation of the highly soluble N-carbonyloxy-D-galactosamine.

Figure 2. Preparation of N-Carbonyloxy-D-Galactosamine.

Figure 3. Preparation of N-Carbonyloxy-D-Glucosamine.
B. EXPERIMENTAL AND RESULTS

1. Preparation of N-Carbobenzyloxy-Hexosamines

a. Preparation of N-Carbobenzyloxy-D-Galactosamine

The preparation of N-carbobenzyloxy-D-galactosamine is illustrated in Figure 2. Ten mg. of Galm-HCl (76) was dissolved in 0.5 ml. of water and was treated with 9.6 mg. of sodium bicarbonate and 0.02 ml. of benzyl chloroformate (Mann Research Laboratories). After shaking vigorously for one hour, 3 ml. of water were added and excess benzyl chloroformate was removed from the mixture by successive extractions with benzene (3 times, 8 ml. each) and petroleum ether (2 times, 5 ml. each). The aqueous layer was shaken with 0.5 ml. of mixed-bed ion exchange resin\(^1\) until it was free of chloride ion. After filtration, the solution and washings were combined and evaporated to dryness in vacuo, yielding a white crystalline compound which was recrystallized from absolute ethanol. The recrystallized material was obtained in 57 per cent yield, m.p. 179-180\(^\circ\) (corr.); \((\alpha)_D^{25} = +56.1\) (water, c = 2.05). N-Carbobenzyloxy-D-galactosamine yielded the following values, when compared with Gm for reducing sugar: Gm (100 per cent), by arsenomolybdate reducing sugar method (77) 69 per cent; by ferricyanide reducing sugar method (78) 69 per cent.

\(^1\) The resin was prepared by mixing equal volumes of Dowex 50, H\(^+\) and Dowex 1, bicarbonate form (both resins were 20 to 40 mesh, 8 per cent cross-linked). A considerable swelling occurs on mixing the resins and the volume refers to the mixture.
Anal.² Calculated for C₁₄ H₁₉ O₇ N : C 53.57; H 6.11; N 4.47
Found : C 53.70; H 6.18; N 4.42

When the reaction was conducted with 200 mg of Gm, the yield of recrystallized material was 70 per cent. The crystalline material is soluble in water, methanol, hot propanol, hot isopropanol, and hot absolute alcohol. It is insoluble in acetone.

N-Carbobenzyloxy-D-galactosamine can be quantitatively converted to Gm-HCl by dissolving the derivative (0.8 mmoles) in 2N hydrochloric acid (20 ml) and treating with hydrogen at atmospheric pressure in the presence of palladium catalyst (80 mg) in a Parr hydrogenation apparatus. After 2.5 hours the material was removed, filtered, and evaporated in vacuo to a syrup. The syrup was dissolved in water and contained 0.8 mmoles hexosamine by analysis (53).

b. Preparation of N-Carbobenzyloxy-D-Glucosamine

The preparation of N-carbobenzyloxy-D-glucosamine is illustrated in Figure 3. Ten mg of Gm-HCl (Pfanstiehl Laboratories) was dissolved in 0.1 ml of water and was treated with 9.6 mg of sodium bicarbonate, and 0.02 ml of benzyl chloroformate. After the mixture had been shaken for one hour, it was filtered and the solid material washed with benzene and petroleum ether. The compound was recrystallized from 30 per cent acetone. The yields varied from 60 to 70 per cent, m.p. 214°,

\( (\alpha)_{D}^{24} = +75.4 \) (pyridine, c = 3.42) (75)

N-Carbobenzyloxy-D-glucosamine yielded the following values, when compared with Gm for reducing sugar:

² Elementary analyses were performed by the Spang Microanalytical Laboratory, Ann Arbor, Michigan.
Figure 4. Absorbancy of a Solution ($\text{H}_2\text{O}$) of N-Carbobenzyloxy-D-Galactosamine or N-Carbobenzyloxy-D-Glucosamine ($c = 0.498$ mg./ml.).
Figure 5. Optical Density vs. Concentration of N-Carbobenzyloxy-D-Galactosamine ($\lambda = 257.5\mu m$).
Gm (100 per cent); by arsenomolybdate reducing sugar method (77), 40 per cent; by ferricyanide reducing sugar method (78), 60 per cent.

2. Additional Properties

a. Absorption Spectra

Figure 4 illustrates the absorption spectra of either N-carbo-benzzyloxy-D-glucosamine or N-carbobenzyloxy-D-galactosamine. A major peak was observed at 257.5 μm. Molar extinction coefficient = 2.18 x 10² cm² per mole. At 257.5 μm, the optical density is linear with concentration, Figure 5.

b. Chromatography

The Rf values of N-carbobenzyloxy-D-glucosamine and N-carbobenzyloxy-D-galactosamine were compared in a variety of solvents. The values obtained are shown in Table 1. Only in the cases of the borate-treated papers were any significant differences in Rf noted.
### TABLE 1

COMPARISON OF Rf OF N-CARBOBENZYLOXY-D-GALACTOSAMINE AND N-CARBOBENZYLOXY-D-GLUCOSAMINE ON WHATMAN NO.1 PAPER

<table>
<thead>
<tr>
<th>Solvent</th>
<th>N-Carbobenzyl-oxy-Galm</th>
<th>N-Carbobenzyl-oxy-Gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Collidine saturated with H2O</td>
<td>.84</td>
<td>.84</td>
</tr>
<tr>
<td>2. Butanol; ethanol; NH4OH; H2O (40,10,1,49)</td>
<td>.71</td>
<td>.71</td>
</tr>
<tr>
<td>3. Butanol, glacial acetic acid, H2O (4,1,5)**</td>
<td>.72</td>
<td>.72</td>
</tr>
<tr>
<td>4. Butanol, pyridine, H2O (4,1,5)</td>
<td>.74</td>
<td>.74</td>
</tr>
<tr>
<td>5. Ethyl acetate, H2O, pyridine (2,2,1)</td>
<td>.75</td>
<td>.75</td>
</tr>
<tr>
<td>6. Ethyl acetate, H2O, glacial acetic acid (3,3,1)</td>
<td>.61</td>
<td>.61</td>
</tr>
<tr>
<td>7. Butanol ethanol, H2O (3,3,4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) sodium borate treated paper*</td>
<td>.79</td>
<td>.80</td>
</tr>
<tr>
<td>b) boric acid treated paper*</td>
<td>.79</td>
<td>.79</td>
</tr>
<tr>
<td>8. Butanol saturated with saturated boric acid solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) untreated paper</td>
<td>.67</td>
<td>.67</td>
</tr>
<tr>
<td>b) boric acid treated paper*</td>
<td>.62</td>
<td>.67</td>
</tr>
<tr>
<td>9. Butanol saturated with saturated sodium borate solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) untreated paper</td>
<td>.61</td>
<td>.61</td>
</tr>
<tr>
<td>b) sodium borate treated paper*</td>
<td>.54</td>
<td>.74</td>
</tr>
<tr>
<td>10. Butanol saturated with H2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) sodium borate treated paper*</td>
<td>.45</td>
<td>.50</td>
</tr>
<tr>
<td>b) boric acid treated paper*</td>
<td>.56</td>
<td>.64</td>
</tr>
</tbody>
</table>

* Papers were treated by dipping in saturated solutions as indicated and dried before chromatography.

** Upper layer.
C. DISCUSSION

Galm (or Gm) can be reacted with benzyl chloroformate on a 10 mg. scale by a simple procedure which yields a stable, crystalline derivative. The melting point of N-carbobenzzyloxy-D-galactosamine (179-180°) differs from the Gm derivative (214°) and therefore can be used to distinguish Galm from Gm. Furthermore the amino sugar can be regenerated by hydrogenation. The technique described for the preparation of N-carbobenzzyloxy-D-galactosamine employs mixed-bed ion exchange resin for deionization of the reaction mixture. It may therefore prove useful as a general procedure for the preparation of water soluble carbobenzzyloxy derivatives of other amino sugars. The carbobenzzyloxy derivatives fulfills to a major extent the criteria established for a good derivative. The preparation of this derivative from a complex mixture has not been attempted, however, it appears likely that this method could be used for the separation of the amino sugars from amino acids and other contaminants.
PART II

GALACTOSAMINE METABOLISM: THE SYNTHESIS OF GALACTOSAMINE 6-PHOSPHORIC ACID AND N-ACETYLGALACTOSAMINE 6-PHOSPHORIC ACID
A. HISTORICAL REVIEW

Although Galm appears to be widely distributed in nature, there are few reports in the literature concerning its metabolism. Lutwak-Mann (79) demonstrated that animal tissues, yeast and bacteria were capable of utilizing this amino sugar. Cardini and Leloir (80) reported that rat liver extracts converted UDPAGm to UDPAGalm, which was subsequently converted to N-Ac-Galm. Recently, however, it was demonstrated that the product of these reactions is not N-Ac-Galm but N-acetyl-D-mannosamine (68). The mode of synthesis of Galm, therefore, still remains in question.

Evidence has been reported suggesting that the metabolism of Galm is analogous to that of D-galactose. In 1953 Cardini and Leloir (81), demonstrated that Galm could be phosphorylated at C-1 by crude extracts of rat liver and brain. These extracts also phosphorylated D-galactose. Extracts from yeast which had been adapted to galactose catalyzed a similar phosphorylation of both substrates. Whether one or two enzymes catalyzed these reactions was not determined. Recently these authors (82) reported that extracts of various rat tissues would phosphorylate N-Ac-Galm. The position of the phosphate group in this case also appears to be at C-1. A tentative report which implies that Galm and galactose metabolism are not completely analogous was the isolation of an acid-stable Galm-phosphate from cartilage (83). The Galm-phosphate was not characterized to any extent, but its acid stability would suggest that the phosphate group is not located at C-1.

With respect to Gm metabolism, Gm-6-P plays a key role both in the synthesis of Gm and in its dissimilation (84–93). In order to ascertain whether Galm-6-P plays a similar role in Galm metabolism, it was
necessary to prepare this compound in substrate quantities. Part II of these investigations is concerned with the chemical synthesis of Galm-6-P and N-Ac-Galm-6-P.

In order to prepare Galm-6-P, several methods were available which had been used for the preparation of Gm-6-P from Gm. Direct methods of phosphorylation have been used by two groups of workers. Anderson and Percival (94) phosphorylated Gm with metaphosphoric acid, and Asahina and Yamaha (95) used pyrophosphoric acid as the phosphorylating agent. The sugar phosphate was isolated as the crude barium salt by the former workers and as a barium salt of unknown purity by the latter workers. Definitive chemical synthesis of Gm-6-P has been achieved by Maley and Lardy (96) and Anderson and Percival (97). These methods involve the condensation of Gm derivatives with diphenyl phosphorochloridate, and subsequent removal of the protective groups, yielding the desired Gm-6-P. Although these methods are definitive they are difficult to use for the preparation of substantial quantities of the sugar phosphate. A method which utilizes polyphosphoric acid as the phosphorylating agent has been described (98). This method was found to yield Gm-6-P as a stable crystalline product. The procedure is relatively simple and can be used for the preparation of large amounts of the hexosamine phosphate. For these reasons this method was subsequently adapted for the preparation of Galm-6-P from Galm-HCl. Proof that the phosphate group is attached to C-6 was obtained by periodate oxidation studies.

In nature, Galm generally exists as the N-acetyl derivative. The chemical synthesis of N-Ac-Galm-6-P was therefore attempted. Acetylation was accomplished by the use of a modified acetic anhydride procedure, generally found applicable to the N-acetylation of the hexosamines (76).
Although Galm has been shown to be acetylated by a pigeon liver enzyme and AcCoA (99), this enzyme appeared to be non-specific since aromatic amines were more effectively acetylated (100). In the present work enzymatic acetylation of Galm-6-P in the presence of AcCoA was studied in Neurospora crassa and various tissue extracts (rabbit). These extracts previously considered to be specific for Gm-6-P (92) were as effective in the acetylation of Galm-6-P. Fractionation of the fungal enzyme did not result in separation of the two activities. Whether a single enzyme catalyzes the acetylation of the two substrates is not yet known.

In Figure 6, are represented the structural formulae of Gm-6-P, Galm-6-P, and their N-acetyl derivatives.
D-Glucosamine 6-Phosphoric Acid (Gm-6-P)  
D-Galactosamine 6-Phosphoric Acid (Galm-6-P)

N-Acetyl-D-Glucosamine 6-Phosphoric Acid (N-Ac-Gm-6-P)  
N-Acetyl-D-Galactosamine 6-Phosphoric Acid (N-Ac-Galm-6-P)

Figure 6. Structural Formulae of the Hexosamine Phosphates.
Figure 7. Chemical Synthesis of Galactosamine 6-Phosphoric Acid.
B. EXPERIMENTAL AND RESULTS

1. Polyphosphoric Acid Method for the Preparation of Galactosamine 6-Phosphoric Acid

The principle of this method is similar to that originally used for the preparation of glucose 6-phosphate (101). The reaction is shown in Figure 7. Galm-HCl is treated with polyphosphoric acid followed by HBr hydrolysis. The resulting Galm-phosphate is precipitated as the barium salt and readily crystallized as the free acid after treatment with mixed-bed ion exchange resin.

Two hundred and forty grams of polyphosphoric acid (Victor Chemical Works; ortho equivalent 115 per cent) were stirred in an ice bath while 24 gm. of ice were slowly added. When the temperature of the mixture was below 5°, 120 gm. of finely powdered Galm-HCl (76) were added. The mixture was stirred at room temperature for 1 hour and at 37° for 15 hours. Ice water (600 ml.) was added and the solution was adjusted to pH 6.5 to 7.0 by the addition of approximately 300 gm. of sodium carbonate. Ice water (1200 ml.) was immediately added, and, after standing in ice for 4 to 5 hours, the mixture was filtered and the residue washed with small quantities of ice water. The combined washings and filtrate (approximately 2 liters) were refluxed for 18 hours with 300 ml. of 48 per cent hydrobromic acid solution. The dark mixture was treated twice with 15 gm. of Darco G-60 carbon (10 minute reflux each) and yielded a light yellow filtrate. The solution was neutralized with excess barium carbonate and filtered, and the crude barium salts were precipitated from the filtrate by the addition of 3 volumes of 95 per cent ethanol. After centrifugation, the barium salts
were washed three times with 70 per cent ethanol. Three extractions of
the residue were performed (100 to 200 ml of water each time) with vigour-
ous stirring for periods of 1 hour each. The turbid extract was clari-
fied by the addition of a minimal volume of water and yielded a yellow or
orange solution. Color and inorganic contaminants were removed by shak-
ing the solution for 2 hours with 75 ml of mixed-bed ion exchange resin.\(^1\)
Since removal of the colored material at this stage was critical, it was
occasionally necessary to filter the mixture and add small amounts of
fresh resin to the solution. After filtration and washing of the resin,
the filtrate (about 900 ml) yielded negative tests for barium, chloride,
and phosphate ions. Acetone was added (from 1 to 3 volumes) until the
appearance of a faint but persistent turbidity; Galm-6-P crystals formed
immediately. The yields of first crop material were 5.5 gm of Galm-6-P.
Addition of acetone to the mother liquors gave about 0.1 gm of Galm-6-P.
The crystalline compound can be used directly for enzyme studies. Re-
crystallization for analysis\(^2\) was effected by dissolving the compound in
water and adding acetone until the appearance of a faint turbidity. The
samples were dried in vacuo at room temperature over phosphorous pentoxide.

Calculated for C\(_6\)H\(_{14}\)O\(_8\)N\(_2\)P: C 27.81, H 5.45, N 5.41, P 11.95

Found : C 27.90, H 5.46, N 5.40, P 11.94

2. **Properties of Galm-6-P**

Inorganic phosphate was not detected in the crystalline material
and only one component was observed when Galm-6-P was examined by paper
chromatography and paper ionophoresis. Saturated solutions of Galm-6-P
in water were about 0.005 M at room temperature, and solutions were un-
stable above pH 7. Galm-6-P decomposed at approximately 200°; [\(\alpha\)\(_D\)\(^{25}\) = +57.8
Figure 8. Crystals of Galm-6-P from Water-Acetone Mixture; (x 25).
Figure 9. X-ray Powder Diffraction Patterns of Galm-6-P and Gm-6-P.
(equilibrium value; 0.1 per cent solution in water). When equimolar concentrations of Galm-6-P and Galm were compared by various colorimetric methods, the values obtained (Galm 100 per cent) were as follows:
ferricyanide reducing sugar method (78), 74 per cent; arsenomolybdate reducing sugar method (77), 100 per cent; and acetylacetone hexosamine method (53), 100 per cent.

The crystalline form and X-ray powder diffraction patterns are indicated in Figure 8 and Figure 9.

3. Periodate Studies with Galm-6-P

In order to verify the position of the phosphate group, the chemically synthesized Galm-phosphate was subjected to periodate oxidation studies. In these experiments, Galm, and the crystalline barium salt of glucose 6-phosphate were used as model compounds. Periodate uptake was measured by the arsenite titrimetric method (93,102).

The solutions contained the following in 25 ml.: Galm-HCl 50 µmoles, acetate buffer (pH 4.5) 5 mmoles, and sodium periodate 400 µmoles. The oxidations were carried out at pH 4.5 at 2°C in the dark. These conditions were previously established by Jeanloz and Forchielli (103) as optimal for periodate oxidation of the hexosamines. The results obtained with Galm, G-6-P, and Galm-6-phosphate are presented in Figure 10. It should be noted that oxidation of Galm-6-P may be predicted to occur either in the open chain form or in the hemiacetal form of the compound. G-6-P may be used as a model compound to illustrate this point, Figure 11. If oxidation proceeds in the open chain form (A), 4 moles of periodate would be consumed and 1 mole of glycolaldehyde-phosphate produced. These results can be obtained only if the phosphate
Figure 10. Periodate Oxidation of Galm, Galm-P and G-6-P.
CHO
H-C-OH
HO-C-H
H-C-OH
H-C-OH
CH₂OPO₃⁻

\[ \text{CHO} \quad \text{H-C-OH} \quad \text{HO-C-H} \quad \text{H-C-OH} \quad \text{H-C-OH} \quad \text{CH₂OPO₃⁻} \]

\[ 4 \text{IO}_4^- \rightarrow 4 \text{HCO}_2\text{H} + \text{CHO} \quad \text{CH₂OPO₃⁻} \]

A.

CHO
H-C-OH
H-C-OH
HO-C-H
H-C-OH
H-C
CH₂OPO₃⁻

\[ \text{CHO} \quad \text{H-C-OH} \quad \text{HO-C-H} \quad \text{H-C-OH} \quad \text{H-C} \quad \text{CH₂OPO₃⁻} \]

\[ 3 \text{IO}_4^- \rightarrow 2 \text{HCO}_2\text{H} + \text{CHO} \quad \text{H-C-O-C-H} \quad \text{CH₂OPO₃⁻} \]

B.

Figure 11. A - Oxidation of G-6-P in Open Chain Form.
B - Oxidation of G-6-P in Hemiacetal Form.

CHO
H-C-NH₃⁺
HO-C-H
HO-C-H
H-C-OH
CH₂OPO₃H⁻

\[ \text{CHO} \quad \text{H-C-NH₃⁺} \quad \text{HO-C-H} \quad \text{HO-C-H} \quad \text{H-C-OH} \quad \text{CH₂OPO₃H⁻} \]

\[ \text{Ac}_2\text{O}, \text{pH 6-7} \quad \text{or} \quad \text{AcCoA-Acetylase} \]

\[ \text{Galactosamine 6-Phosphoric Acid} \quad \text{N-Acetylgalactosamine 6-Phosphoric Acid} \]

Figure 12. N-Acetylation of Galactosamine 6-Phosphoric Acid.
is attached to C-6. If oxidation proceeds in the hemiacetal form (B), 
3 moles of periodate would be consumed and 1 mole of 2-formyl-3-phospho-
glyceraldehyde produced when the phosphate is attached to C-6. An 
analogous situation exists with Galm-phosphate.

As indicated in Figure 10, the rate of oxidation of Galm-
phosphate parallels that of Galm, but the net consumption of periodate 
after 24 hours is equivalent to that obtained with G-6-P, e.g., 4 μmoles 
of periodate per μmole of Galm-phosphate. Thus oxidation proceeds in 
the open chain form. Formaldehyde was isolated as its dimedon deriv-
ative (104) from the Galm reaction mixture, but none was detected in the 
G-6-P, or Galm-phosphate reaction mixtures.

Definitive evidence that Galm-phosphate is the corresponding 
6-phosphate would require the isolation of glycolaldehyde phosphate, the 
expected phosphorylated product of periodate oxidation. Mixtures con-
taining the following components in 50 ml. of solution were prepared: 
0.1 mmole of the substance to be oxidized, 0.8 mmole of sodium metaper-
iodate, 10 mmoles of acetate buffer (pH 4.54). Oxidations were conducted 
for 24 hours at 2-3°C with G-6-P, Galm, and sodium α-glycerophosphate. 
After removal of periodate and iodate with barium hydroxide, the barium 
salts of organic phosphates were precipitated with three volumes of cold 
ethanol. The barium salts were converted to sodium salts by treatment 
with Dowex 50, Na+ and aliquots of the phosphorylated products were frac-
tionated on Dowex 1, Cl- form resin (105) by gradient chromatography. The 
fractions were analyzed for glycolaldehyde (106) and for organic phosphate, 
and in both cases the peaks coincided. Further, the peaks obtained from 
the oxidation of G-6-P, and Galm-phosphate coincided with that obtained 
from the oxidation of α-glycerophosphate.
The results obtained with G-6-P and Galm-phosphate suggest that oxidation occurs in the open chain form and agree with studies of other investigators on fructose 6-phosphate and ribose 5-phosphate (107).

4. Preparation of N-Acetyl-Galactosamine-6-Phosphoric Acid

The procedure used for the N-acetylation of Galm-6-P was a modification of that previously described for the N-acetylation of the hexosamines (76). The reaction is shown in Figure 12.

A mixture containing 3.9 mmoles of Galm-6-P, 2.4 mmoles of NaHCO₃, 9 ml. of water, and 1.2 ml. of methanol was vigorously stirred in an ice bath while 0.32 ml. of acetic anhydride was added. The mixture was stirred at 0-1° and maintained at pH 6 to 7 (titration with 10 per cent NaOH solution) for 90 minutes. The solution was passed through 50 ml. of Dowex 50, H⁺ resin (20 to 40 mesh) and concentrated in vacuo to about 20 ml. Traces of Galm-6-P were removed by passing the solution through a column containing 50 ml. of Dowex 1, acetate³ form resin (200 to 400 mesh). The resin was washed successively with 200 ml. of 0.20N acetic acid and 200 ml. of water. The N-Acetyl-Galm-6-P was eluted from the column with 0.10 N H₂SO₄, 20 ml. per fraction). Fractions 18 to 21 which contained the N-acetyl compound, were combined; sulfate appeared in fraction 41. The solution was treated with Ag₂CO₃ to remove traces of chloride ion, filtered, and passed through 50 ml. of Dowex 50, H⁺ resin to remove silver.

³ The complete conversion of the resin from the chloride form (as it is purchased) to the acetate form is very difficult. Despite prolonged treatment of the resin with sodium acetate and acetic acid, well beyond the point where chloride elution is undetectable, it was found that sulfuric acid eluted small quantities of chloride which frequently appeared in the fractions containing N-Ac-Galm-6-P.
ion from the solution. The combined filtrate and washings were
lyophilized to yield 1.0 gm. of white, hygroscopic solid. Occasionally,
a syrup was obtained, in which case water was added, and the material
was again lyophilized.

Calculated for C₉H₁₆O₉NP:  C 31.90, H 5.64, N 4.65, P 10.78.
Found:  C 32.16, H 5.54, N 4.62, P 10.68.

The optical rotation of N-Ac-Galm-6-P was (α)²⁵ D = +48.4
(equilibrium value; 1 per cent solution in 0.05 M sodium acetate, final
pH 4.5).

The final product was analyzed by a nitrous acid-anthrone
reaction (108) and was found to contain less than 0.1 per cent Galm-6-P.
At equimolar concentrations, the following colorimetric values were
found for N-Ac-Galm-6-P compared with N-Ac-Galm (100 per cent): arseno-
molybdate reducing sugar (77) method, 82 per cent; ferricyanide (78)
reducing sugar method, 66 per cent; modified Morgan-Elson method for
N-acetylhexosamine (58), 51 per cent.

5. Enzymatic N-Acetylation of Galm-6-P (See Figure 12)

The enzymatic acetylation of Gm-6-P by fungal, bacterial and
mammalian tissue extracts has been reported (92). These enzymes appeared
to be specific for Gm-6-P since they were inactive with a variety of
substrates including Gm and Galm. Since Galm usually exists in nature
as its N-acetyl derivative, a study of its enzymatic acetylation was
pertinent. As will be demonstrated, extracts previously shown to be
specific for Gm-6-P (92), also acetylate Galm-6-P. In an attempt to deter-
mine whether or not a single enzyme was catalyzing the reactions, fungal
<table>
<thead>
<tr>
<th>Fractionation Step</th>
<th>Specific Activity**</th>
<th>Gm-6-P*</th>
<th>Galm-6-P*</th>
<th>Gm-6-P/Galm-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>6.7</td>
<td>4.0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>2. Protamine</td>
<td>25.3</td>
<td>17.2</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>3. Ca₃(PO₄)₂ gel</td>
<td>30.0</td>
<td>18.1</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>4. Heat, pH(60°, 4.8)</td>
<td>110.0</td>
<td>108.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>5. Mixed bed resin, lyophilize</td>
<td>160.0</td>
<td>120.0</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>6. Alumina Cγ gel</td>
<td>456.0</td>
<td>456.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>7. (NH₄)₂SO₄, 50-70%</td>
<td>704.0</td>
<td>700.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

** Specific activity was defined as micromoles of acetylhexosamine formed per mg of protein per hour at 37°.

* Product calculated as the free sugars. See text for explanation.

Extracts (Neurospora crassa) were purified and the specific activities (μmoles product formed/mg protein/hour) of the various fractions were compared with either Gm-6-P or Galm-6-P as substrate. The organism was grown, and the acetylating enzyme purified according to the procedure previously described (92).

Incubation mixtures contained the following: Galm-6-P or Gm-6-P, 4 μmoles; AcCoA, 1 μmole; potassium phosphate buffer pH 6.6, 5 μmoles; and enzyme. Final volume was 0.175 ml. Incubations were carried out for 15 minutes at 37°, and the substrates were adjusted to the
pH of the reaction mixture just before use. The reaction was stopped by heating for 3 minutes in a boiling water bath and after removal of protein by centrifugation, an aliquot was removed for analysis (58).

Table 2 summarizes the data obtained on the purification studies. The products formed are presented as N-Ac-Galm or N-Ac-Gm, not as the respective phosphate esters. The Morgan-Elson method (58) yields less color with the phosphates than with the free sugars, but no attempt was made to correct for this difference since phosphatases are present in the extract. The values obtained are therefore considered as minimal. It is possible to conclude that Galm-6-P is as effectively acetylated by the fungal extracts as is Gm-6-P. No indication of separation of activities was obtained after 100-fold purification of the enzyme(s). Whether or not the "acetylase" is a single enzyme cannot be decided by these data.

**TABLE 3**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Substrate Acetylated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gm-6-P*</td>
</tr>
<tr>
<td></td>
<td>µmole**</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.065</td>
</tr>
<tr>
<td>Liver</td>
<td>0.156</td>
</tr>
<tr>
<td>Heart</td>
<td>0.00</td>
</tr>
<tr>
<td>Brain</td>
<td>Trace</td>
</tr>
<tr>
<td>Muscle</td>
<td>&quot;</td>
</tr>
<tr>
<td>Lung</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* Product calculated as the free sugars. See text for explanation.
** Moles of acetylated sugar formed per reaction mixture.
Table 3 summarizes the data obtained with various extracts of rabbit tissues. Extracts were prepared by homogenizing 5 gm. of fresh tissues with 20 ml. of 0.10 M Tris buffer pH 7.4. After centrifugation at 2000 x g for 10 minutes, the residues were discarded. The incubation mixtures contained the following in a final volume of 0.175 ml.: extract from 12.5 mg. of fresh tissue; Galm-6-P or Gm-6-P, 4 μmoles; AcCoA, 1 μmole; potassium phosphate buffer pH 6.1, 5 μmoles. The mixtures were incubated for 30 minutes at 37°.

The results indicate, as observed with the fungal extracts, that rabbit tissues acetylate Galm-6-P as effectively as Gm-6-P. It should be stressed that the animal tissue extracts like the fungal extracts do not acetylate aromatic amines, amino acids, Gm or Galm. The problem of whether the acetylation is carried out by one or more enzymes in the animal extracts has not been resolved.
C. DISCUSSION

Galm-phosphate is a typical dipolar ion and is not appreciably absorbed by ion exchange resins at pH 5 to 6. By taking advantage of this property, charge contaminants were removed and the purified compound readily crystallized. Proof of structure of the hexosamine phosphate was obtained by periodate oxidation studies. The oxidation of G-6-P was studied simultaneously as a model compound. Oxidation was shown to proceed in the open chain form, since 4 moles of periodate were consumed per mole of sugar phosphate oxidized. This was further substantiated by the isolation of glycolaldehyde phosphate from the reaction mixtures. Therefore, it is possible to assign to Galm-phosphate, the structure Galm-6-P.

The isolation of TDPAGalm (109) and the evidence obtained for the phosphorylation of Galm or N-Ac-Galm at C-1 has suggested that Galm metabolism is analogous to that of galactose (110-113). However, in the experiments presented, it was shown that Galm-6-P is acetylated as effectively as Gm-6-P, both by fungal and mammalian extracts. Whether the acetylations were carried out by two distinct enzymes, or by one enzyme was not determined. If separation of the enzymes had occurred, it would be apparent that Galm-6-P is an intermediate in Galm metabolism. Since separation did not occur, this question remains unanswered. In this regard, it might be mentioned that this is the first demonstration of the N-acetylation of Galm by an enzyme(s) which appears to have narrow specificity, in contrast, to the enzyme described by Chou and Soodak (99). Galm-1-P has as yet, not been shown to be enzymatically acetylated. The
results presented on the enzymatic acetylation of Galm-6-P, and the tentative report that an acid stable Galm-phosphate is present in cartilage (83), implies that the 6-phosphates may be of metabolic significance and that Galm and galactose metabolism are not completely analogous.
PART III

METABOLISM OF D-GLUCOSAMINIC ACID
A. HISTORICAL REVIEW

The sugar acids, D-gluconic and D-glucuronic acids, and their phosphate esters, have been established as important intermediates in hexose metabolism. On the other hand, the corresponding hexosaminic acids have received little attention. D-Glucosaminic acid has not yet been isolated from natural sources despite an early report suggesting that an aminotetroxyacproic acid was a component of chondroitin sulfuric acid. The Gmic H⁺ was apparently formed as a result of the experimental conditions used (8). Also erroneous was the report that aminoglucuronic acid was a component of chondroitin sulfuric acid (9). Recently, however, an aminohexuronic acid has been identified as a component of the V₁ antigen (114). The exact configuration of this substance has not been established.

The earliest study of Gmic H⁺ metabolism was carried out by Imaizumi in 1937 (115). He fed Gmic H⁺ to dogs and rabbits and reported the isolation of lactic acid, oxalic acid and acetic acid from the urine. By growth of bacteria in the presence of Gmic H⁺, he isolated lactic and succinic acids from the culture fluid. However, the interpretation of these results is difficult, due to the lack of experimental detail. In 1941, Lutwak-Mann (79) reported that the addition of hexosamines to various tissue slices or suspensions of microorganisms caused an increase in O₂ consumption, accompanied by acid formation and ammonia production. Oxygen consumption in these systems preceded ammonia formation. Lutwak-Mann postulated that the amino sugars were first converted by oxidation into some product, presumably an acid which was then deaminated. Gmic H⁺, suggested as a possible intermediate, was not utilized by her systems, nor could she find evidence for its formation.

-43-
In 1957, Imanaga (116), working with whole cells or cell-free extracts of *Pseudomonas fluorescens*, reported that Gm was oxidized to Gm H⁺. Although this appeared to be the first report on the biological formation of Gm H⁺, the significance of this reaction is not clear, since Gm H⁺ was not further utilized by the whole cells or the cell-free extracts. The enzymatic reaction was not characterized with regard to specificity, but it resembles the glucose oxidase type reaction of *Penicillium notatum*. The latter is known to be highly specific for glucose (117).

The following section of this dissertation contains the results of studies on the metabolism of Gm H⁺ by an unidentified bacterium which utilizes this compound as a sole source of carbon. The first step in the dissimilation of Gm H⁺ was found to be its conversion to equivalent amounts of 2-keto-3-deoxy-D-gluconic acid (KDG) and ammonia. The reaction is shown in Figure 13.

Recently, Imanaga (118) has isolated an unidentified organism from soil, which utilized Gm H⁺ as a sole source of carbon and nitrogen. His results (see Discussion) also suggest that Gm H⁺ is metabolized via the KDG intermediate.
Figure 13. Enzymatic Conversion of D-Glucosaminic Acid to 2-Keto-3-Deoxy-D-Gluconic Acid.
B. MATERIALS

Unless indicated otherwise, all materials were commercial products.

The Qmic H⁺ used in the following studies was prepared from Gm-HEL by oxidation with HgO according to the procedure of Pringsheim and Ruschmann (119) or was obtained commercially (Krishell Laboratories). The commercial material was twice recrystallized after treatment with charcoal.

Calcium 2-keto-D-gluconate and calcium 5-keto-D-gluconate were obtained from the Northern Regional Research Laboratories. Potato phosphatase was prepared according to an unpublished procedure of Kornberg.

I would like to thank the following individuals for their generous gifts of the indicated compounds: L- and D-serine, and L- and D-threonine from Dr. H. N. Christensen (Department of Biological Chemistry, The University of Michigan); 2-keto-3-deoxy-6-phospho-D-gluconic acid from Dr. W. A. Wood (Department of Dairy Science, University of Illinois); mixed calcium α- and β- glucometasilicaglates and calcium β- glucometasilicaglate from Dr. W. M. Corbett and Dr. G. Machell (British Rayon Research Association); and sodium α-ketobutyrate from Dr. H. R. Garner (Department of Biological Sciences, Purdue University).

Preparation of Barium Phosphoenolpyruvate

\[ (C_3H_2O_6P \cdot 3/2Ba \cdot 2H_2O \text{ M.W. } 407) \]

Barium phosphoenolpyruvate was prepared according to an unpublished procedure of Kornberg and Pricer with minor modifications.
A 3-neck, 500 ml. round-bottom flask, fitted with a drying tube, a dropping funnel and a mercury-sealed stirrer was used. The flask was placed in a salt-ice bath and 11.0 gm. of sodium pyruvate (dried over H₂SO₄ in vacuo for approximately 30 hours) and 50 ml. of cold quinoline⁴ were added and the mixture allowed to equilibrate for a few minutes.

Fifteen ml. of POCl₃ (freshly opened or redistilled) were added dropwise while the mixture was stirred. Stirring was rapid enough to permit heat dissipation without splattering the pyruvate above the liquid level. If local heating occurred, the mixture rapidly turned brown and was discarded. The mixture was stirred for 3 hours, during which time it gradually turned a brownish color. The ice-salt mixture was removed and the flask surrounded with a 20-25° water bath. After five hours of continuous stirring, the mixture became very dark and quite viscous. The flask was surrounded with ice-water and the material was dissolved in 400 ml. of cold 30 per cent ethanol. The solution was transferred to an Erlenmeyer flask and 1 liter of approximately 0.12 N HCl was added. After a few minutes the flocculent precipitate was filtered off and discarded. At this point the material was either stored overnight in the cold or placed directly on the charcoal column.

Two hundred and thirty gm. of unground Nuchar C charcoal were suspended in 1.8 liters of 0.1 N HCl and the mixture was briefly boiled. The suspension was slurried into a column about 10 cm. in diameter. The

⁴ Fischer Q-34, C.P. natural quinoline was used. Preparations attempted with synthetic Eastman-Kodak quinoline were unsuccessful, e.g., reaction mixtures quickly formed a dark brown material which resulted in poor yields. Quinoline was refluxed for 2 hours over BaO (1 gm/100 ml) and then fractionally distilled in vacuo.
bottom of the column contained glass wool, under a bed of sand. Several inches of water above the sand prevented disturbance of the surface while the charcoal was poured in. The surface of the charcoal was covered with glass wool, celite, and then sand and the charcoal was finally washed with 0.1 N HCl until the filtrate was free of ultra-violet absorbing material.

The crude reaction mixture was adsorbed on the charcoal and the column was washed with 5 to 6 liters of 0.1 N HCl. Elution was performed with 0.1 M ammonium formate, pH 8.5 (25.8 ml. of reagent formic acid and approximately 660 ml. of 1 M NH₄OH diluted to 6 liters). The flow-rate of the column was 50 ml. per minute and 500 ml. fractions were collected. PEP was present in fractions 8 through 12; PEP was determined by measuring ultra-violet absorption at 212 and 230 mμ (dilutions were made in 0.01 N HCl). The 212/230 ratio is 1.8 - 2.6 when PEP is present.

The eluted material usually contained between 11,000-11,500 μmoles. The fractions were combined (pH 4-5) and the barium salt prepared by titration to a phenolphthalein end point with barium hydroxide, followed by precipitation of the barium salt with 0.8 volume of 95 per cent ethanol. The flocculent precipitate was collected by centrifugation and was washed successively with 70 per cent ethanol, 100 per cent ethanol and ether. The precipitate was dried in vacuo over CaCl₂ and paraffin and contained about 8500 μmoles of PEP. Free pyruvate was not detectable and the product contained less than 4 per cent inorganic phosphate.

The crude barium salt (4.2 gm.) were dissolved in 150 ml. of 0.1 N HCl and 1 M Na₂SO₄ was added in slight excess. The BaSO₄ was
removed by centrifugation and washed with water. The combined supernatant fluid and washings were adjusted to pH 8.0 with NH₄OH, followed by adsorption on 320 ml. of Dowex 1, Cl⁻ x 8 (200-400 mesh). The resin was previously washed free of ultra-violet absorbing materials with 0.01 N HCl and 0.05 M KCl solution.

In the original procedure, the PEP was eluted from the column with 0.01 N HCl and 0.05 M KCl solution. In these studies however, the PEP was not detected in the filtrate although 22.4 l. of this solvent were used. The eluting solution was therefore changed (fraction 71) to 0.05 N HCl and 0.05 M KCl solution and the PEP was found in fractions 77-88. PEP was precipitated as the barium salt as described above; recovery of PEP from the column was quantitative. This material was 85 per cent pure as assayed with pyruvate phosphokinase (121).
C. ANALYTICAL METHODS

1. Enzymatic Phosphorylation

Phosphorylation was determined by measuring acid formation manometrically (120) or ADP formation spectrophotometrically (121). In phosphorylation reactions with ATP, the additional acid group formed for each molecule of substrate phosphorylated, in the presence of NaHCO₃ resulted in the evolution of an equivalent of CO₂ which was measured manometrically.

The Warburg flasks contained the following components (in μmoles) in a final volume of 3.0 ml.: sidearm, 0.1 ml. or 0.2 ml. of crude extract; mainvessel, ATP, 20; MgCl₂, 10; Gmic H⁺ adjusted to pH 7.6, 10. Control flasks were identical except that they did not contain Gmic H⁺. Control values were subtracted from the values obtained when substrate was present.

In the spectrophotometric procedure, ADP formation was measured enzymatically by the following series of reactions.

1. phosphoenolpyruvate + ADP $\xrightarrow{\text{pyruvic kinase}}$ pyruvic acid + ATP

2. pyruvic acid + DPNH $\xrightarrow{\text{lactic dehydrogenase}}$ lactic acid + DPN⁺

Since PEP, lactic dehydrogenase, pyruvic kinase, and DPNH are added in excess, reaction 1 is the rate limiting step and thus depends on the concentration of ADP. This assay will be referred to as the "PEP assay".

Incubation mixtures contained the following components (in μmoles) in 0.6 ml.: Gmic H⁺ adjusted to pH 7.4, 2; ATP, 4; MgCl₂, 2; potassium phosphate buffer, pH 7.4, 15; and extract. Control tubes did not contain Gmic H⁺. After incubation at 37° for various intervals of time, the reaction was stopped by placing the tubes in boiling water for 3 minutes.
Figure 14. Standard Curve for 2-Keto-3-Deoxy-D-Gluconic Acid.
Figure 15. Standard Curve for Glucosaminic Acid.
Aliquots of the reaction mixture were added to cuvettes which contained the following (in \textmu moles) in 3.0 ml.: FEP, 1; MgCl$_2$, 10; potassium phosphate buffer, pH 7.4, 100; DPNH, 0.25; 0.025 mg. lactic dehydrogenase (Worthington Biochemicals, contains 20,000 units per mg.; a unit of activity is that causing a change in optical density of 0.001 per minute). Pyruvic kinase is present as a contaminant of the lactic dehydrogenase. The decrease in optical density at 340 m\textmu was measured in a Beckman model DU spectrophotometer. FEP, prepared as the barium salt, was converted to the potassium salt as previously described. This could also be accomplished by shaking with Dowex 50, K$^+$ (200 to 400 mesh).

2. \textit{\alpha-Keto Acid}

\textit{\alpha-Keto acid} was measured as the semicarbazone according to the procedure of MacGee and Doudoroff (122). To 1 ml. of sample (containing not more than 0.8 \textmu mole of \textit{\alpha-Keto acid}) was added 1 ml. of semicarbazide reagent (1 per cent semicarbazide hydrochloride and 1.5 per cent sodium acetate trihydrate). After incubation for 15 minutes at 37°, the tubes were removed from the water bath and 3.0 ml. of H$_2$O were added. Semicarbazone formation was measured at 250 m\textmu. The standard curve obtained with KDG is shown in Figure 14.

3. \textit{Ammonia}

Ammonia was determined by the Conway diffusion procedure (123).

4. \textit{Glucosaminic Acid}

Gmic H$^+$ was determined by a ninhydrin method (124). The standard curve obtained with Gmic H$^+$ is shown in Figure 15. Standards were prepared with approximately equal buffer concentration as that present in
the incubation mixtures. Ammonia was removed from aliquots of the incubation mixtures by the addition of 1 ml. of 0.01 N NaOH and evaporation to dryness over P₂O₅ in vacuo. The dry residue was redissolved in 1 ml. of H₂O and again evaporated to dryness. The latter step was repeated several times and resulted in complete removal of ammonia. Finally, the tubes were treated with ninhydrin in the usual manner (124).

5. **Pyruvic and α-Ketobutyric Acids**

These substances were determined by the direct method of Friedemann and Haugen (125) with the alcohol modification of Sayre and Greenberg (126) to prevent turbidity.

6. **Enzyme Assay**

In early experiments the following mixtures were routinely used (in μmoles): Gmic H⁺ adjusted to pH 8.0, 10; potassium phosphate buffer, pH 8.0, 15; and enzyme in a final volume of 0.125 ml. In later experiments, the incubation mixtures were supplemented with PLP, 0.016 μmoles and ME, 2 μmoles. Routine enzyme incubations were performed for 5 minutes at 37°. The reaction was stopped by heating the incubation mixtures for 3 minutes in a boiling water bath. Controls included a tube with heat inactivated enzyme and a tube without enzyme. 1 unit of enzyme was defined as the quantity which yields 1.0 μmole of α-keto acid in 5 minutes under the above conditions. The specific activity was defined as the number of units of enzyme per mg. of protein.

7. **Protein Determination**

Protein was estimated by a nephelometric micromethod (127).
D. EXPERIMENTAL AND RESULTS

1. Isolation of a "Bacterium" which Utilizes D-Glucosaminic Acid as Its Sole Carbon Source

A solution of Gmic \( H^+ \) (0.005 M) was allowed to remain uncovered on a laboratory desk top for one week, after which time turbidity developed which appeared to be due to bacterial growth. 1 ml. of the contaminated solution was transferred to 100 ml. of growth medium described below. After 24 hours at room temperature and an additional 16 hours at 37° on a rotary shaker, the turbid solution was streaked on agar plates. Incubation for 24 hours at 37° yielded single white colonies which were transferred to agar slants and stored at 4°. The organism, which has not yet been identified as to genus and species, possesses the following characteristics: a rod-shaped, gram-negative, aerobe, non spore-former and non motile. Dr. Haynes of the Northern Regional Research Laboratories has indicated that this organism is not a Pseudomonad. It possesses the following characteristics on differential media:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Agar Medium</td>
<td>+ hemolysis</td>
</tr>
<tr>
<td>Tellurite</td>
<td>slight growth, slight reduction</td>
</tr>
<tr>
<td>MacConkey’s</td>
<td>- growth</td>
</tr>
<tr>
<td>SS</td>
<td></td>
</tr>
<tr>
<td>Gram-Positive Medium</td>
<td></td>
</tr>
</tbody>
</table>

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5 We are grateful to Dr. W. Callahan (University of Michigan) and Dr. W. C. Haynes and Dr. A. Jeanes (Northern Regional Research Laboratories) who performed the morphological and differential media studies on the unidentified organism.

-55-
6.5% NaCl Medium - growth
Dextrose " acid and gas after 5 days
Lactose, mannitol, sucrose or starch " - growth
Methylene Blue " no reduction
Gelatin " no liquefaction
M.R. and V.P. " - growth
Citrate " + growth
Pigment Production -
Indole -

The organism grows well at room temperature on slants containing tryptone, liver extract, yeast extract and glucose, or on tryptone, yeast extract, and 0.1 per cent potassium gluconate. It also grows well on chemically defined media with glucose as the sole carbon source. A culture of this organism has been deposited at the Northern Regional Research Laboratories and has been designated as NRRL-P-826. In the remainder of this dissertation, NRRL-P-826 is referred to as the "bacterium" or the "organism".

2. Growth of Bacteria for Experimental Studies

The growth medium used in these studies consisted of the following (gm. per liter): NH₄Cl, 2.0; Na₂HPO₄, 6.0; KH₂PO₄, 3.0; NaCl, 5.0; MgSO₄, 0.1; and Gmic H⁺, 2.5. The inorganic salts, dissolved in 900 ml. H₂O, and Gmic H⁺, dissolved in 100 ml. of H₂O, were autoclaved separately at 15 lb. for 15 minutes. Prior to use, the Gmic H⁺ solution was aseptically added to the inorganic salts solution. For the maintenance of stock
cultures and isolation procedures, 2 per cent Bacto-Agar was added to the above medium.

Cells were grown for 24 hours at 37° on a New Brunswick Rotary Shaker, 240 R.P.M. A flask containing 25 ml. of growth medium was inoculated from an agar slant and the contents of the flask used to inoculate 1 liter of medium. The organism was harvested at 4° by centrifugation in a Servall centrifuge at 7000 x g for 15 minutes and washed twice with 0.15 M KCl. Approximately 2.5 gm. of wet weight of cells were obtained per liter of growth medium.

3. Substrate Effect on Oxygen Consumption

Respiration was studied manometrically in the Warburg respirometer using standard procedures (128). The following substrates were tested for their ability to stimulate oxygen uptake: D-glucosaminic acid, D-glucosamine, D-glucose, D-gluconic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-arabinose and D-ribose. The bacteria were suspended in 0.15 M KCl and diluted to 41 per cent transmission at 660 m\(\mu\) in an Evelyn colorimeter.

The results are shown in Figure 16. Of the various sugars tested only D-gluconic acid and Gm\(\mathrm{ic}\) \(\mathrm{H}^{+}\) yielded any detectable stimulation of oxygen consumption. D-gluconic acid was approximately 33 per cent as effective as Gm\(\mathrm{ic}\) \(\mathrm{H}^{+}\). The sugars which gave only a trace or no activity are indicated.
Figure 16. Substrate Utilization by Whole Cells. The sidearm of the Warburg flask contained 10 μmoles of substrate; the centerwell, 0.2 ml. of 7 N KOH absorbed in filter paper; the mainvessel, 1.5 ml. of 0.1 M potassium phosphate buffer, pH 7.4, and 1 ml. of cellsuspension. Final volume, 3.2 ml.
4. Phosphorylation of D-Glucosaminic Acid (by Crude Extracts)

Crude cell-free extracts catalyzed a transfer of phosphate from ATP when Gmic H⁺ was used as substrate. Crude extracts were prepared by suspending approximately 2.5 gm. wet weight of cells in 8 ml. of 0.15 M KCl. Eight gm. of fine glass beads⁶ were added and the cells were disrupted by mechanical shaking (129) for 1 minute. In order to maintain low temperature the container was shaken for 30 seconds, cooled and the process repeated. The suspension was centrifuged at 16,000 x g for 10 minutes and the supernatant fluid used for the phosphorylation studies in the manometric assay. For the PEP assay, the debris was further extracted with 8 ml. of 0.15 M KCl and the supernatant fluids were combined.

The results of the manometric assay (Figure 17) and the PEP assay (Table 4) indicated that in the presence of Gmic H⁺, a phosphorylation reaction was catalyzed by the crude extracts. The manometric assay is time consuming and was subsequently discarded in favor of the more rapid PEP assay.

⁶ Superbrite glass beads, No. 115 regular, Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.
Figure 17. Phosphorylation by Crude Extracts as Measured by the Manometric Assay. The Warburg flasks contained the following components (in μmoles): sidearm, 0.1 ml. or 0.2 ml. of crude extract; main vessel, ATP, 20; NaHCO₃, 75; MgCl₂, 10; Gm H⁺ adjusted to pH 7.6, 10. Final volume 3 ml.
TABLE 4

PHOSPHORYLATION BY CRUDE EXTRACTS
AS MEASURED BY PEP ASSAY

The reaction mixtures contained the following components (in μmoles): Gmic H⁺ adjusted to pH 7.4, 2; ATP, 4; MgCl₂, 2; potassium phosphate buffer, pH 7.4, 15; and extract. Final volume 0.6 ml. Incubated at 37° for the indicated time intervals, and analysed for ADP as described. Activity was expressed as the μmoles ADP formed per incubation mixture.

<table>
<thead>
<tr>
<th>ml. of crude extract</th>
<th>μmoles 15 minutes</th>
<th>μmoles 30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04</td>
<td>0.45</td>
<td>0.90</td>
</tr>
<tr>
<td>0.02</td>
<td>0.21</td>
<td>-</td>
</tr>
</tbody>
</table>

5. Phosphorylation of D-Glucosaminic Acid;
Requirement for an Intermediate Step(s)

The results obtained in the preceding section indicated that a phosphorylation was taking place in the presence of Gmic H⁺, crude extract and ATP. However, by ammonium sulfate fractionation of the crude extract, it was noted that at least two protein fractions were necessary for the phosphorylation reaction. The results indicated that Gmic H⁺ was converted to some intermediate which was subsequently involved in the phosphorylation reaction.

Crude extracts were prepared as previously described. All operations were conducted at 1-2°.
Step 1: - 1 ml. of a suspension of aged calcium phosphate gel (130) containing 16 mg. of solid per ml. was added to 5 ml. of the crude extract. After standing for 15 minutes with occasional stirring, the gel was removed by centrifugation and discarded (Calcium phosphate gel fraction).

Step 2: - 5 ml. of the above supernatant fluid were fractionated with ammonium sulfate. The mixtures were allowed to stand fifteen minutes with occasional stirring before centrifugation at 16,000 x g for fifteen minutes in a Servall centrifuge. The precipitates, designated as AS fractions, were dissolved in 0.1 M potassium phosphate buffer, pH 6.6.

\( \text{AS}_1 \) fraction (0.65 ml.) was obtained on addition of 1.13 gm. of ammonium sulfate; \( \text{AS}_{II} \) (0.70 ml.) on addition of 0.29 gm. of ammonium sulfate to the supernatant solution of the \( \text{AS}_1 \) fraction; \( \text{AS}_{III} \) (0.65 ml.) by the addition of 0.63 gm. of ammonium sulfate to the supernatant fluid of the \( \text{AS}_{II} \) fraction, and \( \text{AS}_{IV} \) (0.45 ml.) by the addition of 0.67 gm. of ammonium sulfate to the \( \text{AS}_{III} \) supernatant fluid. The fractions were analyzed for kinase activity and the results are shown in Table 5.

The \( \text{AS}_1 \) fraction exhibited some activity, but only 26 per cent of that present in the calcium phosphate gel fraction. The \( \text{AS}_{II} \), \( \text{AS}_{III} \), \( \text{AS}_{IV} \) fractions exhibited no detectable kinase activity (occasionally trace amounts of activity were present in the \( \text{AS}_{II} \) fractions). When \( \text{AS}_1 \), \( \text{AS}_{II} \), \( \text{AS}_{III} \), and \( \text{AS}_{IV} \) were combined, 89 per cent of the original activity was attained. These findings indicated that Gmio H+ was not directly phosphorylated but was apparently converted to some intermediate which subsequently was involved in the phosphorylation reaction.
TABLE 5

SEPARATION OF KINASE ACTIVITY BY
AMMONIUM SULFATE FRACTIONATION

The reaction mixtures contained the following components (in μmoles): Gmic H+ adjusted to pH 7.4, 2; ATP, 4; MgCl₂, 2; potassium phosphate buffer, pH 7.4, 15; and 0.005 ml. of the AS fractions. The incubation time was 15 minutes at 37°. Phosphorylation activity was measured by the PEP assay as described. Activity was expressed as the μmoles of ADP formed per reaction mixture.

<table>
<thead>
<tr>
<th>Ammonium Sulfate Fractions</th>
<th>μMoles</th>
<th>% Recovery of Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS_I</td>
<td>0.15</td>
<td>26</td>
</tr>
<tr>
<td>AS_II</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>AS_III</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>AS_IV</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>AS_I + AS_II + AS_III + AS_IV</td>
<td>0.55</td>
<td>89</td>
</tr>
</tbody>
</table>

This tentative conclusion was substantiated by the experiments indicated in Table 6. The results demonstrated that kinase activity was present only in the AS_I fraction and established the presence in the AS_III fraction of an enzyme(s) which converted Gmic H+ to a substance which subsequently participated in the phosphorylation reaction.
TABLE 6

EVIDENCE FOR AN INTERMEDIATE REACTION BEFORE PHOSPHORYLATION

The complete system contained the following components (in μmoles): Gmic H⁺ adjusted to pH 7.4, 2; ATP, 4; MgCl₂, 2; potassium phosphate buffer, pH 7.4, 15; and 0.005 ml. of AS₁ and 0.005 ml. of AS₃. Final volume 0.6 ml.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1st Incubation*</th>
<th>2nd Incubation*</th>
<th>μMoles Phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>complete</td>
<td>not performed</td>
<td>0.47</td>
</tr>
<tr>
<td>2.</td>
<td>omit ATP and AS₃</td>
<td>ATP and AS₃</td>
<td>0.00</td>
</tr>
<tr>
<td>3.</td>
<td>omit ATP and AS₁</td>
<td>ATP and AS₁</td>
<td>0.41</td>
</tr>
<tr>
<td>4.</td>
<td>omit AS₃; add boiled AS₃</td>
<td>not performed</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*1st incubation was performed at 37° for 15 minutes. The reaction was stopped by heating for 3 minutes at 100°. The 2nd incubation (15 minutes at 37°) followed the addition of the indicated components to the cooled tubes. The reaction was stopped as described and ADP determined by the PEP assay. Activity was expressed as the μmoles of ADP formed per reaction mixture.
6. **Enzymatic Conversion of D-Glucosaminic Acid to 2-Keto-3-Deoxy-D-Gluconic Acid (Figure 13)**

a. **Preliminary Studies**

Preliminary studies on the nature of the substance resulting from the action of AS_{III} on Gmic H^{+} indicated that an α-keto acid and ammonia were produced.

Incubation mixtures contained the following components in μmoles: Gmic H^{+} adjusted to pH 7.4, 2; potassium phosphate buffer pH 7.4, 15; and enzyme (AS_{III} fraction); final volume 0.6 ml. Incubations were carried out for 30 minutes, the reaction stopped by heating in boiling water for 3 minutes and aliquots removed for the following studies:

- **Semicarbazone formation**: aliquots when reacted with semicarbazide yielded a typical semicarbazone absorption spectra of the α-keto acids with an absorption maximum at 250 μμ (122).

- **Quinoxaline formation**: aliquots when reacted with o-phenylenediamine yielded a typical quinoxaline absorption spectra of the α-keto acids with a ratio of 330 μμ to 360 μμ of 1.5 (131).

- **Ammonia formation**: ammonia formed in the reaction mixture was detected by the Conway diffusion technique (123).

- **Paper chromatography**: chromatographic studies indicated that the α-keto acid behaved in an identical manner with that of an authentic sample of 2-keto-3-deoxy-D-gluconic acid. The following solvent systems (132) were used: Solvent 1, methanol, ammonia, water, (6,1,3); Solvent 2, methanol, formic acid, water, (80,15,5); and Solvent 3, propanol, formic acid, water, (6,3,1). Ascending chromatography was performed with Solvent 1 for 13 hours. Descending chromatography was performed with Solvents 2...
and 3 for 7 hours. In all cases Whatman No. 1 paper was used. In preliminary studies, alkaline silver nitrate spray (133), semicarbazide spray (134), and o-phenylenediamine spray (131), were used to detect the keto acid. Since the o-phenylenediamine spray appears to be more specific for the keto-deoxy-compounds (132), (135), it was subsequently used. Authentic KDG was prepared from KDPG by the action of potato phosphatase. The results are shown in Table 7. Both the R$_f$ of the enzymatically prepared keto acid and that of authentic KDG were identical in the above solvent systems. In Solvent 3, 2-keto-D-gluconic acid has an R$_f$ of 0.42, thus differing from the unknown keto acid and KDG. Further, the color development of the 2-keto-3-deoxy-hexonic acids when sprayed with the o-phenylenediamine spray appears to be characteristic. Both enzymatically prepared keto acid and KDG revealed a yellow spot after spraying and drying

TABLE 7

<table>
<thead>
<tr>
<th>Solvent No.</th>
<th>Authentic KDG</th>
<th>Enzymatically Prepared KDG</th>
</tr>
</thead>
<tbody>
<tr>
<td>R$_f$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>.89</td>
<td>.88</td>
</tr>
<tr>
<td>2.</td>
<td>.68</td>
<td>.68</td>
</tr>
<tr>
<td>3.</td>
<td>.59</td>
<td>.59</td>
</tr>
</tbody>
</table>

at room temperature. No color develops with 2-keto-D-gluconic acid until the paper is heated. After heating, a greenish spot develops for 2-keto-gluconic acid which subsequently turns to a purple color after standing
at room temperature. Enzymatically prepared keto acid and authentic KDG became greenish-yellow after heating and turned orange-red and finally to red on standing.

b. **Enzymatic Preparation of 2-Keto-3-Deoxy-D-Gluconic Acid**

In order to fully establish the identity of the keto acid it was enzymatically prepared and subsequently crystallized as the calcium salt.

The enzyme was prepared from cells harvested from 2 liters of growth medium as previously described with the following modifications. After removal of the AS fraction (0-40 per cent ammonium sulfate fraction) the concentration of ammonium sulfate was increased to 70 per cent and the precipitate obtained dissolved in 9.0 ml. of potassium phosphate buffer, 0.1 M, pH 6.6. This fraction was used for the preparation of the α-keto acid from Gmic H⁺. The incubation mixture consisted of the following components: 8.6 ml. of enzyme, 2.7 gm. of Gmic H⁺ adjusted to pH 7.4, and 260 ml. of 0.1 M potassium phosphate buffer, pH 7.4. Final volume of incubation mixture 860 ml. After 2 hours incubation at 37°, 91 per cent of the Gmic H⁺ had been converted to the ketonic acid. The reaction mixture was acidified with H₃PO₄ to pH 2-3 and passed through 100 ml. of Dowex 50, H⁺ (200 to 400 mesh). The eluate was neutralized to pH 7.2 with Ca(OH)₂, the calcium phosphate removed by centrifugation and the supernatant fluid concentrated in vacuo (132). The pH was maintained at 7.2 by addition of Ca(OH)₂ until no change could be detected over a period of several hours (the pH slowly decreases, presumably due to opening of the lactone ring). After concentration to a syrup and trituration with methyl alcohol, the
syrup solidified. The yield of calcium salt was 2.47 gms. (94 per cent of the total ketonic acid). The calcium salt was redissolved and concentrated to a syrup which could be crystallized by the addition of several drops of acetone. If too much H₂O was present, crystallization was difficult. The calcium salt was recrystallized for analysis and dried in vacuo over P₂O₅ for several days at room temperature. [α]⇧D²⁵ = -29.2° (6.15 per cent solution in H₂O). The results of elementary analyses² were:

Calculated for CaC₁₂₀₁₂H₁₈.₁/₂H₂O: Ca 9.94; C 35.73; H 4.75.

Found: Ca 10.17; C 35.55; H 4.77.

c. Reduction of Enzymatically Prepared 2-Keto-3-Deoxy-D-Gluconic Acid to the Glucometaseaccharinic Acids

For the further characterization of the keto acid, it was desirable to convert it to a compound of known chemical structure. This was accomplished by conversion of the keto acid by borohydride reduction to a mixture of the α- and β- isomers of the glucometaseaccharinic acids. Figure 18.

500 mg. of the calcium salt of the enzymatically prepared keto acid were dissolved in H₂O and converted to the potassium salt by shaking with Dowex 50, K⁺ (200-400 mesh). After removal of the resin by filtration, the filtrate was evaporated (all evaporationes were performed in vacuo) to a syrup and dissolved in 5 ml. of H₂O and 10 ml. of 0.1 M borate buffer pH 8. The flask was placed in an ice bath, 400 mg. of NaBH₄ added, and the contents stirred. After 1 hour, the solution gave a negative test for semicarbazone formation (122). The reaction mixture was removed from the ice bath and excess borohydride was destroyed by the addition of 8 N HCl to pH 1. After concentration to dryness, borate was
removed as the methyl ester by repeated addition and evaporation of methyl alcohol and 1 drop of 8 N HCl. In order to remove cations, the solution was finally treated with Dowex 50, H⁺ and brought to dryness several times until only a faint chloride test was obtained. The syrup which contains a mixture of the α- and β- glucometasaccharinic acids in the form of their lactones was dissolved in H₂O and aliquots were removed for paper chromatographic studies.

Descending paper chromatography was performed with ethyl acetate, acetic acid, and H₂O (10, 1.3, 1) (136) as solvent on Whatman No. 1 paper. Chromatograms were developed with hydroxylamine-ferric chloride spray (137). An authentic mixture of the α- and β- glucometasaccharinates and the reduced acids prepared form the keto acid gave identical Rf's. β-isomer 0.29, α-isomer, 0.33. The reduction with NaBH₄ does not appear to be stereospecific since both substances appeared to be present in equal amounts.
1. Calcium β-Glucometasaccharinate (Authentic)  
2. Calcium β-Glucometasaccharinate (Prepared by Reduction of 2-Keto-3-Deoxy-D-Gluconic Acid)

Figure 19. X-ray Diffraction Patterns.
In order to form the calcium salts of the mixed α- and β-
glucometasaccharinates prepared from the keto acid, Ca(OH)₂ was added
with stirring until no further decrease in pH (approximately at pH 8)
could be detected over a period of several hours (decreases in pH are
presumably due to opening of the lactone ring). Dry ice was added to re-
move any excess calcium as CaCO₃. The solution was evaporated to a
syrup which solidified to a white solid after trituration with ethyl
alcohol. The β-isomer was fractionally crystallized from the mixture
(138) by dissolving the crude calcium salts in a minimum amount of hot
H₂O. Crystallization begins several hours after cooling of the solution.
The material was subsequently placed at 4° for approximately 12 hours,
and the crystals collected by centrifugation and washed several times
with small amounts of ice-cold H₂O. For X-ray analysis the material was
recrystallized. The yield of β-isomer was 50 mg. This represents a 20
per cent yield if both the α- and β- isomers were present in equimolar
concentrations. Chromatography of the recrystallized calcium salt in-
dicated that only the β-isomer was present. The X-ray powder diffraction
patterns of authentic calcium β-glucometasisaccharinate and that obtained
by the above process are shown. Figure 19.

7. Purification and Properties of
Glucosaminic Acid Dehydroase

a. Purification of Glucosaminic Acid Dehydroase

The enzymatic conversion of Gmic H⁺ to KDG is similar to the
reactions catalyzed by the well known serine and threonine deaminases or
dehydrases. The enzyme has thus been designated Gmic H⁺ dehydroase.
Crude extract: - Unless indicated otherwise, all operations were conducted between 0-4°, and centrifugations carried out at 16,000-18,000 x g for 10 minutes. Freshly harvested cells, or cells frozen and stored at -18° (usually not more than one month old) were used. Approximately 2.5 gm. of wet weight of cells were suspended in 9 ml. of 0.15 M KCl and disrupted as previously described. After centrifugation, the debris was extracted twice with 6 ml. of 0.15 M KCl and the supernatant fluids combined.

Protamine step: - 5 ml. of a 2 per cent solution of protamine sulfate were added to 19 ml. of the crude extract, and after stirring, the mixture was allowed to stand for 10 minutes and then centrifuged. The precipitate was discarded and the supernatant fluid was dialyzed against 0.01 M KCl for 3-6 hours (dialysis for 18 hours did not result in loss of activity). After dialysis the turbid solution was centrifuged and the precipitate discarded.

DEAE - cellulose\(^7\) step: - The DEAE-cellulose was washed with a solution containing 0.01 M potassium phosphate buffer, pH 7.0 and 0.02 M KCl until the supernatant fluid was pH 7.0. Before use, the DEAE-cellulose was washed with water several times. 22 ml. of the dialyzed protamine supernatant fluid containing the enzyme were added to 12 ml. of DEAE-cellulose. The mixture was occasionally stirred for 15 minutes and then centrifuged at 2000 x g. Occasionally larger quantities of the cellulose were needed to adsorb the bulk of the enzyme. After adsorption of at least 75 per cent of the enzyme, the supernatant fluid was discarded and

---

\(^7\) Diethylaminoethyl-cellulose, Type 20, Brown Co.
the cellulose washed several times with ice-cold water. Elution was
carried out by extraction of the cellulose (two times) with 20 ml. of a
solution containing 0.01 M potassium phosphate buffer, pH 7.1 and 0.08
M KCl. The eluates were combined and used for the subsequent studies.

The enzyme purification is summarized in Table 8. The final
enzyme preparation was 9-fold purified. The purification varied from
9 to 13-fold, yield from 65 to 80 per cent and specific activity from
700 to 1200. The enzyme was stored at -18° and exhibits variable sta-
bility. Losses of activity have occasionally been observed after sev-
eral days but in some instances, preparations have remained stable for
as long as a month.

TABLE 8

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Units</th>
<th>Specific Activity x 10</th>
<th>Per Cent Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>8100</td>
<td>12.7</td>
<td>100</td>
</tr>
<tr>
<td>Protamine step</td>
<td>7900</td>
<td>56.3</td>
<td>98</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>5280</td>
<td>117.2</td>
<td>65</td>
</tr>
</tbody>
</table>

b. Properties of Glucosaminic Acid Dehydrase

i. Stoichiometry of the Reaction.--As indicated in Table 9, equivalent
amounts of α-keto acid and ammonia were formed, while an equimolar
amount of GmIC H⁺ disappears.
TABLE 9

STOICHIOMETRY OF D-GLUCOSAMINIC ACID DEHYDRASE REACTION

The reaction mixtures contained (in µmoles): Gmic H⁺ adjusted to pH 7.8, 40; potassium phosphate buffer, pH 7.8, 60; and 6.4γ of protein. Final volume 0.5 ml. The incubation time was 15 minutes at 37°.

<table>
<thead>
<tr>
<th>µmoles/incubation mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosaminic acid</td>
</tr>
<tr>
<td>2-keto-3-deoxygluconic acid</td>
</tr>
<tr>
<td>NH₄⁺</td>
</tr>
</tbody>
</table>

ii. Specificity of Glucosaminic Acid Dehydrase.--The Gmic H⁺ dehydrase reaction is analogous to those reactions catalyzed by the serine and threonine dehydrases. That is, serine by the action of serine dehydrase is converted to pyruvic acid and ammonia and similarly threonine is converted to α-ketobutyric acid and ammonia. Since Gmic H⁺ is structurally related to these amino acids, the action of Gmic H⁺ dehydrase on these substrates were tested.

The results are shown in Table 10. L-serine and D-serine exhibited approximately 1 per cent of the activity obtained with Gmic H⁺. No activity could be detected with L-threonine and 2 per cent with D-threonine. Thus, of the substrates tested, Gmic H⁺ dehydrase appeared to be relatively specific for Gmic H⁺.
TABLE 10

SPECIFICITY OF D-GLUCOSAMINIC ACID DEHYDRASE

The reaction mixtures contained: substrates adjusted to pH 7.8, 10 μmoles; potassium phosphate buffer, pH 7.8, 15 μmoles; and 1.6 γ protein. Final volume 0.125 ml. The incubation time was 15 minutes at 37°.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>μmoles Keto Acid* Formed</th>
<th>% Activity Compared to Glucosaminic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucosaminic acid</td>
<td>4.56</td>
<td>100.0</td>
</tr>
<tr>
<td>L-serine</td>
<td>0.04</td>
<td>0.8</td>
</tr>
<tr>
<td>D-serine</td>
<td>0.06</td>
<td>1.0</td>
</tr>
<tr>
<td>L-threonine</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>D-threonine</td>
<td>0.10</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* μmoles formed per incubation mixture.

iii. Effect of pH on the Reaction Velocity.—As is indicated in Figure 20, the reaction proceeds at maximal velocity from about pH 7.8 to 8.8.

iv. Effect of Enzyme Concentration.—The reaction rate was linear with protein concentration as indicated in Figure 21.

v. Effect of Incubation Time on the Reaction.—As shown in Figure 22, the reaction was linear with time for at least 20 minutes.

vi. Effect of Substrate Concentration.—The effect of Gmic H⁺ concentration on the reaction velocity is shown in Figure 23. Figure 24 represents the data plotted by the method of Lineweaver and Burk (139). The Km determined by this method is 8.8 x 10⁻³ M.
Figure 20. Effect of pH on the Reaction Velocity. The reaction mixtures contained the following (in µmoles): Gmic H⁺, 20; buffer, 30, PLP, .032; ME, 4; and 0.63γ of protein. The final volume was 0.25 ml. and the incubation time was 5 minutes at 37°. Since Gmic H⁺ has buffering capacity, it must be adjusted before use. The indicated pH is the resulting value of Gmic H⁺ and buffer.
Figure 21. Effect of Enzyme Concentration on Reaction Velocity. The reaction mixtures contained the following (in μmoles): GmH\textsuperscript{+} adjusted to pH 8.0, 10; potassium phosphate buffer, pH 8.0, 15; PLP, 0.016; ME, 2; and protein as indicated. The final volume was 0.125 ml, and the incubation time was 5 minutes at 37°.
Figure 22. Effect of Incubation Time on the Enzyme Reaction. The reaction mixtures contained the following (in µmoles): Glic H⁺ adjusted to pH 8.0, 10; potassium phosphate buffer, pH 8.0, 15; PLP, 0.016; ME, 2; and 1.9γ of protein. The final volume was 0.125 ml, and incubations were carried out at the indicated time intervals at 37°.
Figure 23. Effect of Substrate Concentration on Reaction Velocity. The reaction mixtures contained the following (in μmoles): potassium phosphate buffer, pH 8.0, 15; PLP, 0.016; ME, 2; 0.38γ of protein and the indicated concentration of GmH⁺ (adjusted to pH 8.0). The final volume was 0.125 ml. and incubation time was 5 minutes at 37°C.
Figure 24. Determination of Km by the Method of Lineweaver and Burk.
8. Evidence for Cofactor Requirement

During studies on the Gmic H⁺ dehydrase reaction, it was sometimes found that extracts lost activity on standing. Partial restoration of activity could be obtained by the addition of PLP. It was also found that on further aging of the extract, the stimulation by PLP decreased. However, when a sulfhydryl compound was added with the PLP, the enzymatic activity was significantly increased. Both PLP and GSH have been implicated in the serine and threonine dehydrase reactions (see Discussion). AMP was also implicated in the amino acid dehydrase reactions (140) but did not stimulate activity in the Gmic H⁺ dehydrase reaction.

In order to demonstrate an actual dependence of the reaction on PLP and a sulfhydryl compound, dissociation of the cofactor(s) was attempted by aging. Aging has previously been used to demonstrate that PLP is required by similar enzymes (141, 142).

a. Aging of the Bacterium and the Extract

Cells were harvested, washed in the usual manner and stored at -18° for 6 months. Crude extracts of the aged cells exhibited considerably less Gmic H⁺ dehydrase activity than extracts of fresh cells. When PLP was added the activity of the aged cell extract was stimulated approximately 3-fold. When the crude extract was stored at -18° for approximately one month, activity was not detectable unless both PLP and ME were added. The extract was repeatedly thawed for analysis during this interval and it is not known whether aging or repeated freezing and thawing was responsible for the development of cofactor requirements.
b. **Purification of the Aged Extract**

For the purification of the aged extract, the procedure described previously for the purification of GmIC H⁺ dehydrase was attempted. After protamine treatment of the crude extract, followed by dialysis, the enzyme did not behave as expected and was not adsorbed by the DEAE-cellulose, even after the addition of PLP and ME.

The enzyme was subsequently purified according to the following scheme.

**Crude extract:** - The crude extract was prepared from 2.5 gm. of wet weight of cells as previously described.

**Protamine step:** - 3.8 ml. of a 2 per cent solution of protamine sulfate were added to 15 ml. of the crude extract and after stirring, the mixture was allowed to stand for 10 minutes and then centrifuged. The precipitate was discarded and the supernatant fluid was dialyzed against 0.01 M KCl (6 hours). After dialysis the turbid solution was centrifuged and the precipitate discarded.

**Ammonium sulfate step:** - 16 ml. of the supernatant fluid obtained above was treated with an equal volume of a solution of ammonium sulfate saturated at 1-2°. After standing for 15 minutes with occasional stirring the turbid solution was centrifuged and the supernatant fluid was discarded. The precipitate was dissolved in 8 ml. of 0.1 M potassium phosphate buffer, pH 7.4 and this preparation was used for the studies which follow unless otherwise indicated.

The enzyme purification is summarized in Table 11.
TABLE 11

PURIFICATION OF AGED EXTRACT

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Units</th>
<th>Specific Activity</th>
<th>Per Cent Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1620</td>
<td>34</td>
<td>100</td>
</tr>
<tr>
<td>Protamine step</td>
<td>1052</td>
<td>80</td>
<td>65</td>
</tr>
<tr>
<td>Ammonium sulfate step</td>
<td>865</td>
<td>277</td>
<td>53</td>
</tr>
</tbody>
</table>

c. Effect of AMP, ME and PLP on Gmic H⁺ Dehydrase Activity

As indicated later (see Discussion), PLP, AMP and GSH have been implicated in the serine and threonine dehydrase reactions. The following studies demonstrate that the Gmic H⁺ dehydrase is activated by PLP and ME, while AMP does not appear to be involved.

The results are indicated in Table 12 and demonstrate the dependence of the Gmic H⁺ dehydrase reaction on PLP and ME when aged enzyme is used.

d. Effect of Sulfhydryl Agents on α-Keto Acid Formation

Several sulfhydryl compounds exhibited variable effects on the formation of α-keto acid.

The results in Table 13, demonstrate that ME is most effective in stimulating α-keto acid formation.

e. Effect of Pyridoxal Phosphate and 2-Mercaptoethanol Concentration on the Reaction Velocity

The effect of PLP and ME concentration on the reaction velocity is shown in Figure 25 and 26 respectively.
TABLE 12

EFFECT OF AMP, PLP, AND ME ON α-KETO ACID FORMATION

The reaction mixtures contained (in μmoles): 0.016 M HCl adjusted to pH 8.0, 10; potassium phosphate buffer, pH 8.0, 15; 1.7γ of protein, and the following additions as indicated, PLP, ME, and AMP, 0.016, 2, and 0.13 μ moles respectively. The final volume was 0.125 ml. and incubation time was for 5 minutes at 37°. After stopping the reaction 0.25 ml. of 0.2 M ZnSO₄ and 0.25 ml. of saturated Ba(OH)₂ solution were added in order to remove the nucleotide. All incubation tubes were treated in an identical manner. After centrifugation the supernatant fluids were analyzed for α-keto acid.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>μmoles* α-Keto Acid Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>none</td>
<td>0.01</td>
</tr>
<tr>
<td>2.</td>
<td>PLP</td>
<td>0.02</td>
</tr>
<tr>
<td>3.</td>
<td>ME</td>
<td>0.00</td>
</tr>
<tr>
<td>4.</td>
<td>AMP</td>
<td>0.00</td>
</tr>
<tr>
<td>5.</td>
<td>PLP + ME</td>
<td>0.42</td>
</tr>
<tr>
<td>6.</td>
<td>PLP + AMP</td>
<td>0.00</td>
</tr>
<tr>
<td>7.</td>
<td>ME + AMP</td>
<td>0.00</td>
</tr>
<tr>
<td>8.</td>
<td>PLP + ME + AMP</td>
<td>0.36</td>
</tr>
</tbody>
</table>

* μmoles formed per incubation mixture.
TABLE 13

EFFECT OF SULPHYDRYL AGENTS ON \(\alpha\)-KETO ACID FORMATION

The reaction mixtures contained the following (in \(\mu\)moles); Gmic \(H^+\) adjusted to pH 8.0, 10; potassium phosphate buffer, pH 8.0, 15; PLP, .032; AMP, 0.13; sulphydryl compound, 1; and 6 \(\gamma\) protein (a protamine sulfate supernatant solution, undialyzed). The final volume was 0.125 ml. and incubation time was 5 minutes at 37\(^\circ\). Cysteine hydrochloride and GSH were neutralized before use. The reaction mixtures were treated with 0.25 ml. of 0.2 M ZnSO\(_4\) and 0.25 ml. of a saturated solution of Ba(OH)\(_2\). After centrifugation, aliquots of the supernatant fluid were analyzed for \(\alpha\)-keto acid.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Sulphydryl Compound</th>
<th>(\mu)moles* (\alpha)-Keto Acid Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GSH</td>
<td>0.28</td>
</tr>
<tr>
<td>2.</td>
<td>sodium thioglycollate</td>
<td>0.13</td>
</tr>
<tr>
<td>3.</td>
<td>cysteine</td>
<td>0.03</td>
</tr>
<tr>
<td>4.</td>
<td>2-mercaptoethanol</td>
<td>0.58</td>
</tr>
</tbody>
</table>

* \(\mu\)moles formed per incubation mixture.
Figure 25. Effect of Pyridoxal Phosphate Concentration on Reaction Velocity. The reaction mixtures contained (in μmoles): Gm c H+ adjusted to pH 8.0, 10; potassium phosphate buffer pH 8.0, 15; ME, 2; 1.7γ of protein; and the indicated concentration of PLP. The final volume was 0.125 ml. and incubation time was 5 minutes at 37°.
Figure 26. Effect of 2-Mercaptoethanol Concentration on Reaction Velocity. The reaction mixtures contained (in μmoles): GmC H⁺ adjusted to pH 8.0, 10; potassium phosphate buffer pH 8.0, 15; PLP, 0.032; 1.7γ of protein; and the indicated concentration of ME. The final volume was 0.125 ml. and incubation time was 5 minutes at 37°.
E. DISCUSSION

D-Glucosaminic acid presents an interesting biochemical problem, since this sugar is an amino acid and is related to the hexosamines (D-glucosamine) in the same way that D-gluconic acid is related to D-glucose. In the latter case, the acid is an important intermediate in the metabolism of D-glucose. In order to study the dissimilation of Gmic H⁺, a bacterium which utilized Gmic H⁺ as a sole source of carbon, was isolated by an enrichment technique. This organism was surprising in its lack of ability to oxidize a number of sugars. Of the various sugars tested, only Gmic H⁺ and D-gluconic acid were oxidized, while the following sugars were not: D-glucosamine, D-glucose, D-arabinose, D-ribose, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid. The interesting fact that D-gluconic acid was oxidized (at about 30 per cent of the rate of Gmic H⁺) indicated a possible metabolic relationship between these two sugars and is discussed below in greater detail. It should be noted that, although D-glucose was not appreciably oxidized during the time interval of this experiment, the bacterium will grow on a glucose synthetic medium. This suggests the adaptive nature of this organism.

Since D-glucosamine is known to be metabolized via phosphorylated intermediates, our preliminary studies were based on the hypothesis that Gmic H⁺ was first phosphorylated before further utilization. In fact, it was found that crude, cell-free extracts, catalyzed a phosphorylation in the presence of Gmic H⁺ and ATP. Purification of the crude extract however, demonstrated that two protein fractions were required for phosphorylation. Further study of this phenomenon indicated that
D-glucosaminic acid

2-keto-3-deoxy-D-gluconic acid

Figure 27. Schematic Representation of Proposed Enzymatic Reaction.
Gmic H⁺ was first converted to some intermediate, which was then apparently phosphorylated. The products of enzyme action on Gmic H⁺ were a keto acid and ammonia. Thus the kinase activity originally measured appeared to be dependent on the initial formation of the keto acid.

Preliminary chromatographic studies on the keto acid indicated that it was 2-keto-3-deoxy-D-gluconic acid (KDG). In order to fully characterize the keto acid, it was prepared enzymatically, crystallized as the calcium salt and identified unequivocally as KDG by the following procedures: (1) semicarbazone absorption spectrum, (2) quinoxaline absorption spectrum, (3) paper chromatography, (4) elementary analysis, and (5) conversion to a mixture of α- and β-glucometasaccharinates by NaBH₄ reduction. The α- and β-glucometasaccharinates were chromatographically identical to a known mixture of α- and β-glucometasaccharinates. Final characterization was achieved by isolation of the calcium β-glucometasaccharinate and comparison of its X-ray powder diffraction pattern with the pattern obtained from an authentic sample of the compound.

The enzyme (Gmic H⁺ dehydrase) which catalyzes the conversion of Gmic H⁺ to KDG and ammonia was subsequently purified and its properties studied. The reaction is shown schematically in Figure 27. Stoichiometry studies indicated that equivalent amounts of KDG and ammonia were formed, while an equimolar amount of Gmic H⁺ disappears. The pH optimum of the reaction lies between 7.8 to 8.8. The Km was determined to be about 8.8 x 10⁻³M for Gmic H⁺.
The reaction catalyzed by Gmic $H^+$ dehydrase is analogous to those reactions catalyzed by the serine and threonine dehydrases e.g.,

$$\text{CH}_2-\text{CH}^+ \text{COO}^- \xrightarrow{\text{Serine dehydrase}} \text{CH}_3-\text{C}^- \text{COO}^- + \text{NH}_4^+$$

Serine

$$\text{OH} \quad \text{NH}_3^+$$

Pyruvic acid

$$\text{CH}_3-\text{CH}^- \text{COO}^- \xrightarrow{\text{Threonine dehydrase}} \text{CH}_3-\text{CH}_2-\text{C}^- \text{COO}^- + \text{NH}_4^+$$

Threonine

$$\text{OH} \quad \text{NH}_3^+$$

$\alpha$-ketobutyric acid

In the present case, the enzyme preparation of Gmic $H^+$ dehydrase did not appreciably attack L- and D-serine or L- and D-threonine.

As will be discussed below, PLP and GSH are required for certain of the amino acid dehydrase reactions. During early studies on the Gmic $H^+$ dehydrase reaction, when PLP was included in the reaction mixtures, the production of keto acid was increased. However, dialysis of the enzyme preparation did not result in significant losses in enzyme activity which suggested that the cofactor was not readily dissociable from the enzyme. When extracts were prepared from frozen cells which had been aged over a long period of time (3 to 6 months), the stimulation of enzyme activity by PLP was markedly increased. Aging has previously been used to demonstrate PLP requirements for amino acid dehydrases (141, 142). The cofactor studies are in agreement with the recent findings of Immanaga (118). He demonstrated that cell-free extracts of a soil bacterium, grown on Gmic $H^+$ as the sole source of carbon and nitrogen, will degrade Gmic $H^+$ to an unidentified
keto acid (postulated to be KDG); formation of the keto acid was increased in the presence of ATP and pyridoxal or boiled extract.

In the present studies, it was shown with the aged extract, that a sulfhydryl requirement is necessary in addition to the PLP. Whether the sulfhydryl compound acts as a non-specific reducing agent or has a specific sulfhydryl effect on the enzyme is not known.

The mechanism of the amino acid dehydrase reactions has not been clearly defined and some confusion still remains as to the necessary cofactors. Gale and Stephenson (143) in 1938, studied serine deamination with cell suspensions of E. coli. The reaction proceeded anaerobically, thus distinguishing it from the oxidative deaminases. Loss of activity with aged cell suspensions could be prevented by the addition of phosphate alone, or in combination with various reducing agents such as glutathione, H₂ or formate or by the addition of a boiled suspension or autolysate. The reaction was more clearly defined by Chargaff and Sprinson (144, 145), who studied serine and threonine deamination with E. coli. They identified the products of the deamination as pyruvate and α-keto butyrate and suggested the following mechanism since hydroxyl substitution prevented the reaction:

\[
\begin{align*}
\text{CH}_2-\text{CH}-\text{COO}^- & \xrightarrow{-\text{H}_2\text{O}} \text{CH}_2=\text{C}-\text{COO}^- \\
\text{OH} & \quad \text{NH}_3 ^+ \\
\text{CH}_3-\text{C}-\text{COO}^- & \xrightarrow{-\text{H}_2\text{O}} \text{CH}_3=\text{C}-\text{COO}^- + \text{NH}_4^+ \\
\text{NH}_3 & \quad \text{NH}_2^+ \\
\text{NH}_2^+ & \quad 0
\end{align*}
\]

Their studies with cell-free extracts from rat, mouse, and rabbit tissues, indicated the liberation of appreciable amounts of ammonia from the above substrates after the addition of Mg²⁺ to thoroughly dialyzed solutions. Binkley (146) used cell-free extracts of E. coli and mouse liver to study serine deamination and found that losses of
activity on dialysis could be restored by Mg\(^{++}\), Zn\(^{++}\), or Mn\(^{++}\). Lichstein (147) inactivated the serine and threonine deaminases of *E. coli* and was able to restore activity by the addition of biotin or adenylic acid. When he used a cell-free extract, only yeast extract caused partial reactivation and he suggested that a coenzyme form of biotin present in the yeast extract was involved in the reaction. The *E. coli* dehydrase was purified by Wood and Gunsalus (140) and evidence was presented that one enzyme attacks both L-serine and L-threonine. Both AMP and GSH were required for activity. On the other hand, Metzler and Snell (148) found PLP activation with the D-serine dehydrase of *E. coli*. Umbarger and Brown (149) recently presented evidence for two L-threonine dehydrases in *E. coli* -- the one described by Wood and Gunsalus (140) which requires PLP, AMP and glutathione for maximal activity and a second in which PLP is the cofactor and AMP and glutathione have no effect. The L-serine and L-threonine dehydrases of sheep liver were separated by Sayre and Greenberg (126); they demonstrated each to be a specific enzyme. Dialyzed serine dehydrase was shown to be activated by PLP, AMP and GSH. However, the combined activation of these 3 substances, although greater than any single one alone, were not additive. AMP alone gave the highest activation. Greater difficulty was encountered in demonstrating cofactors with the threonine dehydrase. Only by aging or lyophilization could small amounts of activation by PLP, AMP, and GSH be observed. A recent study of L-threonine dehydrase of the rumen microorganism LCI by Walker (150), demonstrated that PLP was the cofactor in this reaction. PLP doubled the activity of
the reaction, while PLP and GSH increased the activity more than five-fold. Surprisingly, AMP inhibited activation by PLP. Yanofsky (141) and Yanofsky and Reissig (151) demonstrated that PLP was required for activity with specific L-serine, L-threonine and specific D-serine, D-threonine dehydrases obtained from Neurospora. AMP and GSH were inactive in these systems.

The actual function of PLP in the dehydrase reactions has not been completely established. Metzler, Ikawa, Snell (152) have proposed a general mechanism of PLP function in a variety of reactions, based on non-enzymatic model systems. Serine, for example, is non-enzymatically deaminated by pyridoxal and certain metal salts at 100°C (153). The following scheme has been proposed for dehydration of β-hydroxy amino acids and for the desulfhydration of cysteine.

\[ X \quad \text{R} -\text{C} - \text{C} = \text{COO}^- \quad \text{NH}_3^+ \quad \text{M}^{+++} \quad \text{CHO}^- \quad \text{HO}_2\text{C} \quad \text{CH}_3 \quad \text{H}^+ \]

\[ X \quad \text{R} -\text{C} - \text{C} = \text{C} = \text{O} \quad \text{H}^+ \]

\[ X \quad \text{R} -\text{C} - \text{N} \quad \text{H}^+ \]

\[ \text{M}^{+++} \quad \text{CHO}^- \quad \text{HO}_2\text{C} \quad \text{CH}_3 \quad \text{H}^+ \]

\[ \text{M}^{+++} \quad \text{CHO}^- \quad \text{HO}_2\text{C} \quad \text{CH}_3 \quad \text{H}^+ \]

\[ X = \text{OH}, \text{SH} \]

\[ \text{M}^{+++} = \text{Metal} \]
The first step in the above series of reactions is the formation of the Schiff base as its metal chelate from the amino acid and pyridoxal. The metal functions in stabilizing the intermediate Schiff base and also provides an additional electron attracting group in the same direction as the N atom. Dehydration could result by a release of proton at (a) as in II \rightarrow III. If X on the \beta-carbon is an electron attracting group, the sequence of reactions from III \rightarrow V would lead to the release of X as an anion. Finally, hydrolysis would lead to ammonia, \alpha-keto acid and pyridoxal.

A requirement for metal ions by the dehydrases has not been unequivocally established, although there is some evidence that they are necessary (141). Metzler et al. (152) state that perhaps it is possible that a portion of the enzyme protein itself could fill the role played by metal ions in the non-enzymatic reactions. Furthermore, the Schiff base intermediate could still be stabilized (in the absence of metal) by hydrogen bonding (153).
These "β-elimination" reactions include a variety of other enzymatic reactions analogous to the dehydrase reactions with Gmic H⁺, serine and threonine. Examples of several of these reactions are (a) the desulphydration of cysteine (154), (b) tryptophanase reaction, (155) and (c) dehydrochlorination of β-chloro-L-alanine (156). Each of these reactions are known to be stimulated by PLP

(a) desulphydration of cysteine

\[
\text{CH}_2\text{SH} + \text{NH}_3^+ \rightarrow \text{CH}_3\text{C} - \text{COO}^- + \text{NH}_4^+ + \text{H}_2\text{S}
\]

(b) tryptophanase reaction

\[
\text{CH}_2\text{CH}_2\text{CH} - \text{COO}^- + \text{NH}_3^+ \rightarrow \text{CH}_3\text{C} - \text{COO}^- + \text{NH}_4^+ + \text{H}_2\text{O}
\]

(c) dehydrochlorination of β-chloro-L-alanine

\[
\text{CH}_2\text{Cl} + \text{NH}_3^+ \rightarrow \text{CH}_3\text{C} - \text{COO}^- + \text{NH}_4^+ + \text{Cl}^- + \text{H}^+
\]
To return to the problem of Gmic H\(^+\) metabolism, the isolation of free KDG (e.g., not as a phosphate ester) from the Gmic H\(^+\) dehydrase reaction is the first report (157) of the natural occurrence of this compound. As indicated above, Imanaga has reported (118) that Gmic S\(^+\) is converted to an unidentified keto acid (probably KDG). It was of interest, therefore, to note that KDG has recently been identified by Ashwell, Wahba and Hickman (158) as an intermediate in uronic acid metabolism in E. coli.

The 6-phosphate ester of KDG has, however, been known to occur as an intermediate in the dissimilation of glucose by certain Pseudomonas species (Entner-Doudoroff pathway) (122, 159, 160, 161). As indicated in Figure 28, 6-phosphogluconic acid is dehydrated to KDPG which is subsequently cleaved to pyruvic acid and 3-phosphoglyceraldehyde by an aldolase type reaction. This pathway of glucose dissimilation to 3 carbon units represents a different mechanism for the oxidation of glucose, distinct from the glycolytic and the pentose phosphate pathways.

Although the further metabolism of KDG has not been defined in the present studies, preliminary evidence suggests that it may enter the Entner-Doudoroff pathway. In crude extracts, it was shown that a phosphorylation occurs, which is dependent on the initial formation of KDG. If KDG is the substrate for this "kinase", it is probable that KDPG is the product. KDPG could then be cleaved to pyruvic acid and triose phosphate. This possible scheme of degradation of Gmic H\(^+\) is shown in Figure 29, and is supported by the work of Imanaga (118) and Ashwell et al. (158). Imanaga demonstrated that
Figure 28. Entner-Doudoroff Pathway of Glucose Dissimilation.

Figure 29. Proposed Pathway for the Dissimilation of D-Glucosaminic Acid.
Gmic H⁺ is converted to a keto acid and pyruvic acid in the presence of ATP. In the absence of ATP, however, negligible amounts of pyruvic acid were formed, while the total amount of keto acid remained constant. Ashwell et al. (158), studying uronic acid metabolism, reported that KDG is formed from D-altrolic acid and D-mannonic acid. KDG was further metabolized to stoichiometric amounts of pyruvic acid and triose phosphate in the presence of ATP and cell-free extracts.

In this connection, it is important to note that other non-phosphorylated 2-keto-3-deoxy compounds have also been shown to be intermediates in intermediary carbohydrate metabolism. Thus, De Ley and Doudoroff (132), have demonstrated that galactose is metabolized to 2-keto-3-deoxy-D-galactonic acid by extracts of _Pseudomonas saccharophila_. The further dissimilation of this substance to pyruvic acid and triose phosphate is dependent on the presence of ATP. D-arabinose is metabolized by extracts of the same organism to what appears to be 2-keto-3-deoxy-D-arabonic acid (162). This substance is degraded to pyruvic acid and glycolic acid in the presence of DPN⁺.

As previously described, only Gmic H⁺ and D-gluconic acid were effective in stimulating oxygen consumption with whole cells grown on Gmic H⁺. Since Gmic H⁺ and D-gluconic acid are structurally similar, differing only at C-2, the question arises whether a similar pathway exists for D-gluconic acid dissimilation in this organism. Although purified Gmic H⁺ dehydrase did not act on D-gluconic acid, it is possible that D-gluconic acid is phosphorylated to 6-phospho-D-gluconic acid, which subsequently could be metabolized via the Entner-Doudoroff pathway.
Although in the whole cell experiments, D-glucose was not oxidized within the time interval of the experiment, the organism will grow on synthetic medium containing D-glucose as the sole carbon source. This presents the interesting problem of the pathway D-glucose dissimilation by the D-glucose grown cells. Since it is probable that the Entner-Doudoroff pathway does exist in this organism, the problem arises as to whether D-glucose is predominantly metabolized in this manner, or whether this pathway is exclusive for Gmic $H^+$ metabolism and perhaps D-gluconic acid metabolism. A further problem that might be considered with respect to this organism is D-glucosamine metabolism. Can the cells be adapted to D-glucosamine, and if so, is D-glucosamine oxidized to Gmic $H^+$ which could then enter the Entner-Doudoroff pathway? The oxidation of D-glucosamine to Gmic $H^+$ has been described (116), although the Gmic $H^+$ was not metabolized further by this organism. If such a pathway does exist for D-glucosamine dissimilation in the organism used for the present work, this would differ radically from its metabolism via the phosphorylated intermediates, the only pathway known at the present time for the conversion of D-glucosamine to carbon dioxide and water.
SUMMARY
SUMMARY

1. The characterization of small quantities of D-galactosamine has been hampered by the lack of suitable stable, crystalline derivatives. While certain of the Schiff bases have heretofore been regarded as the most satisfactory derivatives, these compounds are relatively unstable and difficult to purify.

The preparation of N-carbobenzyloxy-D-glucosamine, a stable crystalline derivative of D-glucosamine has been previously described, but its application to D-galactosamine was reported to be unsuccessful, since the latter forms a water-soluble derivative. In the present studies, by use of a mixed-bed resin technique, stable, crystalline, N-carbobenzyloxy-D-galactosamine was obtained in good yield. The melting point of N-carbobenzyloxy-D-galactosamine (179°-180°) differs from the D-glucosamine derivative (214°) and can be used for identification purposes. Furthermore, the methods of preparation can be applied to 10 mg. quantities of either sugar. N-carbobenzyloxy-D-galactosamine can be quantitatively converted to the free sugar by hydrogenolysis.

2. Although D-galactosamine is a constituent of a wide variety of biological substances, little is known about its intermediary metabolism. In the case of the closely related sugar, D-glucosamine, the phosphate esters are known to be the principal intermediary metabolites. D-galactosamine 6-phosphoric acid was therefore prepared for enzyme studies. By treatment of D-galactosamine with polyphosphoric acid, followed by acid hydrolysis, crystalline D-galactosamine 6-phosphoric acid was obtained and exhibited the correct elementary analysis. Proof that the phosphate group is attached to C-6 was obtained by periodate oxidation. The
crystalline galactosamine 6-phosphate consumed 4 moles of periodate and the resulting glycolaldehydephosphate was isolated by ion-exchange chromatography.

In nature, D-galactosamine generally exists as the N-acetyl derivative. Chemical synthesis of N-acetyl-D-galactosamine 6-phosphoric acid was accomplished by treatment of an aqueous solution of D-galactosamine 6-phosphate with acetic anhydride. After treatment with ion-exchange resin the product was obtained as a white hygroscopic solid which exhibited the correct elementary analysis. Enzymatic acetylation of D-galactosamine 6-phosphate in the presence of acetyl coenzyme A was studied with extracts of Neurospora crassa and of various rabbit tissues. Previous studies had shown that preparations from these sources are highly specific for D-glucosamine 6-phosphate and do not acetylate D-galactosamine, D-glucosamine, p-nitroaniline, or amino acids. However, these extracts were found to acetylate D-galactosamine 6-phosphate as effectively as D-glucosamine 6-phosphate. The Neurospora enzyme was purified over 100-fold, but the fractionation did not result in separation of the two activities. Whether the acetylation of D-galactosamine 6-phosphate and D-glucosamine 6-phosphate is catalyzed by one or by two enzymes, was not determined.

3. The metabolism of D-glucosaminic acid, an amino acid which is structurally related to the hexosamines, was studied with a bacterium which utilized this sugar as the sole carbon source. Respiration studies indicated that resting cell suspensions of the organism oxidized D-glucosaminic acid and D-gluconic acid (relative rates: 100, 36) but did not consume oxygen in the presence of D-glucose, D-glucosamine, 2-keto-D-gluconic
acid, 5-keto-D-gluconic acid, D-arabinose, or D-ribose. Crude cell-free extracts of the organism catalyzed a phosphorylation reaction in the presence of D-glucosaminic acid, ATP and Mg++. The extract was separated into two fractions, both of which were required for the phosphorylation reaction. A study of these fractions indicated that D-glucosaminic acid was first converted to equimolar quantities of an α-keto acid and ammonia. The α-keto acid was enzymatically prepared, crystallized as the calcium salt and identified as 2-keto-3-deoxy-D-gluconic acid by the following procedures: elementary analysis, chromatography, reduction to a mixture of the α- and β- glucometaseaccharinic acids and fractional crystallization of the β-glucometaseaccharinic acid. The latter compound was finally characterized by comparison of its X-ray powder diffraction pattern with the pattern obtained from an authentic sample of β-glucometaseaccharinic acid.

The enzyme which catalyzed the conversion of D-glucosaminic acid to 2-keto-3-deoxy-D-gluconic acid and ammonia (designated as D-glucosaminic acid dehydrase) was purified and its properties studied. The reaction was found to be dependent on the presence of pyridoxal phosphate and sulfhydryl compounds.
BIBLIOGRAPHY


42. Strange, R. E., Biochem. J., 64, 23p (1956).


60. Tracey, M. V., Biochim. et biophys. acta, 17, 159 (1955).


70. Bergmann, M., and Servas, L., Ber., 64, 975 (1931).


