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Anomers Of D-Galactosamine Benzoates

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ANOMERS OF D-GALACTOSAMINE BENZOATES

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CHAPTER I

INTRODUCTION AND HISTORICAL SURVEY

D-Galactosamine (2-amino-2-deoxy-D-galactose) was the second amino sugar to be isolated from natural sources. In 1914, almost forty years after the discovery of the more common D-glucosamine, Levene and LaForge (62) reported the isolation from chondroitin sulfate of a new amino sugar, which was isomeric with D-glucosamine. The structure of chondrosamine, as it was first named, was not completely determined for another thirty years, when James et al. (32, 33) achieved an unequivocal synthesis of a compound that was identical with the natural product.

Between its discovery and its synthesis, a number of investigators contributed to a knowledge of the nature of D-galactosamine. Although first isolated from chondroitin sulfate extracted from bovine tendon, cartilage, or trachea, more recent work has found small amounts of D-galactosamine widely distributed in such things as β-heparin, certain lipoids, cerebral gangliosides, and submaxillary mucins. However, the substance has been difficult to isolate in sizeable amounts, until the more recent developments in ion-exchange and other methods of column chromatography have made its separation practical. D-Glucosamine, which has been easier to obtain and easier

to crystallize, was taken as the prototype of all the 2-amino-2-deoxy-D-hexoses. However, the biological importance of D-galactosamine was thought to justify an investigation of the properties of a number of its derivatives, which are reported in this dissertation.

The early structural studies on chondrosamine were continued by Levene (59,60), who found that oxidation with bromine formed a 2-aminohexonic acid. He also found that it formed the same phenylosazone as did D-galactose. A Strecker type synthesis of D-galactosamine and its epimer--D-talosamine--from D-lyxose (59) confirmed the configuration of the three, four, five, and six carbons. However these experiments left the configuration of the C-2 amino group undecided.

In 1916 Hudson and Dale (30) prepared the pentaacetates of chondrosamine and were able to separate the anomers by crystallizing the alpha form from an absolute alcohol solution by adding ether. They then measured the specific optical rotations of the two purified forms. Their work proved that Hudson's "2A" values agreed with each other for the acetates of glucose, glucosamine, and chondrosamine. At a considerably later time, Stacey (79) was able to show that acetylation of chondrosamine by acetic anhydride in pyridine produced the alpha pentaacetate, while if zinc chloride were used as the catalyst

only the beta form was produced.

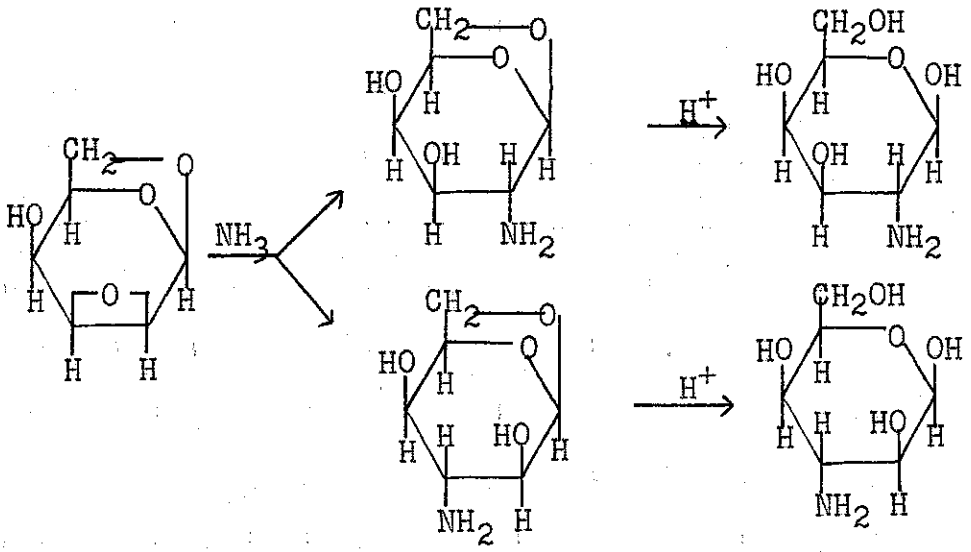
In 1923 Levene (60) isolated the pure alpha and beta chondrosamine hydrochlorides. The first sample isolated (62) had been the alpha form. However, all later preparations, of both the natural and synthetic product, had given the beta anomer, indicating this as one of the few sugars which crystallized as this form. By repeatedly recrystallizing the product from methanol-ethanol-ether, Levene was able to separate the two anomers. The difference in the molecular rotation (α_D x molecular weight) of the two forms was calculated to be 16,485, a value nearly identical with the normal value for the majority of sugars, indicating the samples were probably pure. The accepted values of the specific rotations today are; α anomer $+135^{\circ} \longrightarrow +93^{\circ}$; β anomer $+39^{\circ} \longrightarrow +93^{\circ}$ (in water).

One further insight into the structure of chondrosamine was the degradation of methylated chondrosamine by Levene (61) to prove that it had the pyranose structure. In 1937 Karrer and Mayer (41) identified the products of a lead tetraacetate oxidative degradation of N-carbethoxy-monobenzal-galactosaminic acid ethyl ester hydrochloride. They concluded that the benzylidene group was attached through carbons four and six, rather than through numbers five and six. Their work also identified a 1,4-lactone ring system in N-acetyl-D-galactosaminic acid. By the use

of optical rotatory dispersion they found that the amino and hydroxyl groups on carbons two and three, respectively, were in a trans position.

The final definitive proof of the configuration of the amino group on C-2 in D-galactosamine was provided by James et al.(32,33) in 1945 and 1946. Their synthesis was dependent on the discovery that anhydro rings of the ethylene oxide type, when formed by the alkyl-O-fission of suitable esters (i. e. p-toluene sulfonates), undergo ring scission on either side of the oxygen bridge, when subjected to nucleophilic attack. In each case Walden inversion occurs where the entering anion attaches to the molecule, giving two isomeric derivatives. In the sugars one of the possible isomers usually predominates over the other (22,37,46).

Previous work had shown that chondrosamine must be either 2-amino-D-galactose or 2-amino-D-talose. James et al. (32,33) started with 1,6-anhydro-2-O-mesyl-β-D-galactose and changed it by alkaline hydrolysis to 1,6 : 2,3-dianhydro-β-D-talose. The two anhydro rings were found to differ in stability, with the 1,6-linkage stable toward alkali and broken by acids. Treatment of the dianhydro compound with ammonia, followed by hydrochloric acid, was found to produce chiefly the 2-amino-D-galactose, together with a small amount of 3-amino-D-idose, as illustrated on page 5.



The hydrochloride salt of the D-galactosamine proved to have the same physical constants and X-ray powder photograph as the naturally occurring chondrosamine hydrochloride and gave rise to identical derivatives.

The increasing number of investigations of the chemistry of D-galactosamine and its derivatives in recent years can be attributed to the development of improved methods for isolating the product from natural sources. Even after finding D-galactosamine present in a number of natural products, its percentage was low enough to make difficult a chemical separation from other closely related products. Modern methods of ion-exchange chromatography were first applied to this problem by Gardell (20). He found in 1953 that a column of Dowex 50 (H^+ form) ion-exchange resin would separate D-galactosamine from D-glucosamine, using 0.3 N hydrochloric acid as the devel-

oping solvent. Also separated were the ammonium chloride and other contaminents that were usually present. This method, as refined by Wheat and Davidson (93), has been the best current method for obtaining D-galactosamine hydrochloride from the hydrolyses of chondroitin sulfate.

D-Galactosamine differs from D-glucosamine only in the configuration of the C-4 hydroxyl group. In D-galactosamine it is cis to the C-3 hydroxyl group, and in D-glucosamine it is trans to the C-3 substituent in the Fischer projection formula. With the usual chair form of the hexagonal formula this means that while D-glucosamine has both the C-3 and C-4 hydroxyl groups in an equatorial position, D-galactosamine has the C-3 hydroxyl group equatorial and the C-4 hydroxyl group axial. This axial hydroxyl group makes D-galactosamine the less stable of the two amino sugars. In simple mixtures D-galactosamine can be separated from D-glucosamine by its ability to form a complex with boric acid, because of these cis hydroxyl groups. This fact has been applied mainly in paper chromatography up to the present time.

The use of Schiff bases for the isolation of small quantities of D-glucosamine and D-galactosamine was investigated by Jolles and Morgan (40). They determined the properties of the compounds formed from ten different aldehydes and concluded that the two best condensing agents

were 2-hydroxynaphthaldehyde and 3-methoxy-4-hydroxybenzaldehyde (vanillin). The former, which has the advantage of forming bright yellow crystals, has been used considerably since their work in 1940. More recently the controlled formation of anhydro rings by the alkaline elimination of mesyl and tosyl groups has led to the formation of certain D-galactosamine derivatives from the more readily available D-glucosamine (21,22,35) or from D-lyxose (49). These methods should help to increase the available supply of this rather rare amino sugar.

The synthesis of the pentaacetates of D-galactosamine by Hudson and Dale (30) has been previously mentioned. The five reactive positions of D-galactosamine differ considerably in activity. Experiments have shown that the N-acetyl group is more stable than the O-acetyl groups (79), and a limited amount of reagent goes first to the N-position, or conversely, acyl substituents are removed first from the oxygen positions. In addition, the pyranose form in which the sugars are usually found imparts more activity to the hemiacetal hydroxyl on the first carbon than to the other hydroxyl groups in the molecule. Next in activity is the primary hydroxyl group at C-6, which exceeds that of the secondary alcoholic functions. An understanding of the order of activity of the positions in D-galactosamine helps to explain the synthesis of its various derivatives, which

are given in the following pages of this chapter.

Because D-galactosamine has, in common with the other hexosamines, five reactive positions, most types of synthesis require selective blocking of various positions in the molecule. Since the time of Fischer, acetate groups have been most frequently used in this respect for they are rather easily removed by hydrolysis. Also a factor is the fact that some natural products, including chondroitin sulfate, already contain N-acetyl groups. Benzoyl groups sometimes give considerably more stability to the molecule (70,87) at the cost of slower and less vigorous reactions. They are more difficult to attach or to remove than the acetate groups. The most stable blocking groups are the methyl ethers, and they have been widely used in elucidating the structure of the naturally occurring polysaccharides. Of course, a wide variety of groups have been used to form glycosides at C-1. All of the blocking groups will be mentioned further in connection with specific syntheses.

In recent years carbobenzoxy chloride (benzyloxycarbonyl chloride or benzyloxy chloroformate) has been used as a reagent for attaching a blocking group to the nitrogen atom of an amino sugar or an amino acid (27,89,91). It has the advantage of being easily removed by catalytic hydrogenation, without affecting other groups in the molecule. Other more drastic reagents, such as hydrogen bromide

in glacial acetic acid, also will cleave this group (87). Reyns and Beck (27) in 1957 were the first to prepare N-carbobenzoxy-D-galactosamine. An improved procedure was developed by Smith *et al.* (78), and was the basis for the product prepared for the investigation covered by this report.

Several methods have been developed for placing a halogen atom on the anomeric carbon atom (C-1). These halogen compounds vary considerably in stability, but they are valuable intermediates for the synthesis of a number of derivatives of D-galactosamine. In general, the stability of acetyl glycosyl halides decreases in the order of fluorine, chlorine, bromine, iodine (26), although not all of these have been prepared in the D-galactosamine series. In the D-glucosamine series, the iodide will decompose within a short time, while the fluoride can be subjected to alkaline de-O-acetylation without loss of halogen. The bromide has often represented the best compromise between reactivity and stability.

Glycosyl halides have been prepared by the following methods (26):

1. Treatment with liquid hydrogen halide. This is the only way to make the fluoride compound.

2. Treatment with hydrogen halide in dry ether. This has been used mostly for the chloride compound.

3. Treatment with hydrogen halide in glacial acetic acid. Emil Fischer (15) discovered this widely used method. Acetic anhydride can also be used as a combined solvent and acetylating agent (23).

4. Treatment with phosphorus pentahalide plus aluminum chloride in chloroform. These reagents cause isomerization with some sugars.

5. Treatment with titanium tetrahalide in chloroform (72).

6. Treatment with acetyl halide (31). This reagent forms the acetyl ester of the halogen compound.

Evidence indicates that, with D-glucosamine, the alpha anomer of the halide is formed by these reactions (31,87). A discussion of this factor as applied to D-galactosamine is given in Chapter II of this report.

Schlubach and Gilbert (77), in 1930, were the first to prepare acetobromo- and acetochlorogalactose, and they also found that the beta anomer could be formed from the alpha by reaction with "activated" silver chloride. The first halogen derivatives of D-galactosamine were described in 1958 by Tarasiejska and Jeanloz (86), who prepared the acetochloro- and acetobromo-compounds as intermediates for the synthesis of some phosphate esters. They found that reaction of either one of the anomers of pentaacetyl-D-galactosamine with hydrogen chloride or hydrogen bromide in glacial acetic acid formed the unstable C-1 halide. Immediate reaction with an alcohol in the presence of

mercuric cyanide or silver oxide changed it into a stable glycoside. In the same year Heyworth and Leaback (28) prepared acetochlorogalactosamine by treatment of an anomeric mixture of D-galactosamine pentaacetates with acetic anhydride-hydrochloric acid reagent. This product was condensed with p-nitrophenol in acetone to yield the beta glycoside, as the acetate ester, which was also de-O-acetylated to give p-nitrophenyl 2-acetamido-2-deoxy-β D-galactoside. Both groups of investigators reported the acetohalogen derivative of D-galactosamine subject to rearrangement on recrystallizing to the 1,3,4,6-tetra-O-acetyl-α-D-galactosamine hydrohalide.

In 1961 Onodera and Komano (71) treated 1,3,4,6-tetra-O-acetyl-2-carbobenzoxy-α,β-D-galactosamine with hydrogen bromide in glacial acetic acid to form 3,4,6-tri-O-acetyl-2-amino-1-bromo-2-deoxy-α-D-galactose hydrobromide. This was confirmed by forming the β-methyl glycoside and finally the N-acetyl derivative, which was identical to an authentic sample, and had the same constants as reported in the literature (86). The same reaction had been used on the corresponding D-glucosamine benzoate ester by Weidmann and Zimmerman (87). In both cases the carbobenzoxy group was cleaved with evolution of carbon dioxide and formation of benzyl bromide. The same acetobromogalactosamine compound was formed in 1963 by Wolfrom et al.

(96) by the direct reaction of acetyl bromide on D-galactosamine hydrochloride. It too was characterized by forming the known β-methyl N-acetyl derivative. The present investigation fills in the gap left in the previous work by a study of the derivatives of the benzoyl esters of bromogalactosamine hydrobromide.

Only a few other glycosides of D-galactosamine have been reported in the literature, except for those used in biochemical preparations, which will be covered in a later section. In 1959 Wolfrom and Yosizawa (101) reacted N-acetyl-D-galactosamine with ethanethiol to form the acyclic diethyl dithioacetal derivative, which changed in aqueous mercuric chloride-mercuric oxide solution to the ethyl-thio-β-D-galactopyranoside. When treated with acetic anhydride in pyridine, the acetal formed the acetate derivatives of both the alpha and beta glycosides with a furanose ring system. Each of the ring structures was confirmed by a periodate oxidation.

In 1960 Kushida and Hayashi (53,54) prepared the ethyl glycosides of N-acetyl-D-galactosamine. By the use of both paper and column chromatography they were able to identify and then to separate the alpha and beta anomers from each other. They also isolated a third component which proved to be an ethyl β-galactofuranoside.

Another phase of the derivatives of D-galactosamine

has been covered by the work of Jeanloz et al. (34,38,39, 80,81,82,83) who in recent years have synthesized all the methyl ethers of this amino sugar. These ethers were characterized in order to help identify the products from the decomposition of methylated polysaccharides obtained from natural sources. The methods used were, in general, the same ones that had been previously used for the corresponding D-glucosamine derivatives. In some cases, though, this was not possible. The difficulties are pointed out in the following quote from Stearns et al. (80) and are confirmed by the information in this dissertation:

> However, side reactions already noticed in the glucosamine series became much more important, because of the change from an equatorial to an axial configuration of the hydroxyl group at C-4, when passing from the D-glucosamine to the D-galactosamine series.

Most of the methyl ethers were made by changing D-galactosamine to the N-acetyl compound, followed by the formation of the alpha methyl glycoside. The remaining carbon three, four, and six hydroxyl groups were then selectively blocked or methylated to form the required derivatives. Methylation was done with dimethyl sulfate and sodium hydroxide in dioxane or with methyl iodide and silver oxide in N,N-dimethylformamide. For example, Stoffyn and Jeanloz (81) synthesized the 3-methyl ether by blocking the four and six positions with a benzylidene

group, methylating the C-3 position, and hydrolytically removing the benzylidene group. This was changed to the free base and characterized by formation of a Schiff base with 2-hydroxynaphthaldehyde. It was also changed into the known 3,4,6-trimethyl ether derivative, which had been reported much earlier by Levene (61).

The 4-O-methyl-D-galactosamine hydrochloride was formed by using James' et al. (32,33) method of forming a D-galactosamine derivative from 1,6 : 2,3-dianhydro-β-talose, which has only the 4-hydroxyl position free for methylation. After methylation, ammonia was used to open the 2,3-epoxide to a D-galactose derivative, then acetic anhydride to acetylate the C-2 amino and the C-3 hydroxyl groups, followed by hydrochloric acid to open the 1,6-ring. Deacetylation led to the required 4-methyl compound. This was confirmed by the same type of derivatives as were used for the 3-methyl compound.

The 6-O-methyl-D-galactosamine hydrochloride was synthesized in two different ways (63). Both routes started with methyl N-acetyl-α-D-galactosaminide. One method was to form the C-6 trityl ether, then the C-3 and C-4 benzoate, followed by removal of the trityl group by hydrolysis, and methylation of the resulting hydroxyl group. The second method involved blocking the C-3 and C-4 positions with an isopropylidene group, methylating the free C-6 hydroxyl,

and removing the blocking group by hydrolysis. Changing to known derivatives completed the identification.

The 4,6-O-methyl derivative was the first of the dimethyl ethers of D-galactosamine to be prepared (82). The methyl N-acetyl-α-D-glycoside was used as starting material. The C-4 and C-6 positions were blocked by reacting with benzaldehyde before placing a p-tolylsulfony group on the third carbon. Acetic acid then removed the benzylidene group and methylation produced the required 4,6-dimethyl derivative. Hydrochloric acid hydrolysis removed all the other protecting groups leaving the 4,6-dimethyl-D-galactosamine hydrochloride, which was characterized as the trimethyl ether, and as the Schiff base of 2-hydroxynaphthaldehyde.

The 3,4-dimethyl ether was completed by starting with 1,6 : 2,3-dianhydro-β-D-talose (38), similar to the synthesis of the 4-methyl compound. However, the C-3 hydroxyl group was found to be more resistant to methylation, so that the required dimethyl compound was produced in low yield.

The last of the dimethyl ethers--3,6-di-O-methyl--of D-galactosamine was synthesized by Stearns et al. (80) in 1961. They first tried to methylate methyl N-acetyl-6-trityl-α-D-galactosaminide, expecting that the 3-methyl would be formed in preference to the 4-methyl derivative.

Results indicated 53% of the 3-methyl and also 32% of the 3,4-dimethyl compound. However, attempts to benzoylate the free hydroxyl in the monomethyl ether gave a low yield of the C-4 benzoate, which was methylated in the C-6 position, after removal of the triphenylmethyl group. Removal of the benzoate blocking group then completed the synthesis. It was noticed that benzoylation of C-4 also resulted in some replacement of the acetamido by the benzamido group. Alkali would also cause the axial C-4 benzoyl group to migrate to the C-6 equatorial primary hydroxyl group. Crystalline derivatives were formed to completely characterize the 3,6-dimethyl-D-galactosamine hydrochloride.

A number of other derivatives of D-galactosamine have been prepared in recent years and will be briefly mentioned. Wolfrom et al. (98) have studied the nuclear magnetic resonance spectra of the acetyl derivatives of D-galactose phenyl- and p-nitrophenylhydrazones, in which they found evidence for an acyclic structure in sugars and for the chelate structure in phenylosazones, as first proposed by Fieser and Fieser (13).

Some sulfur derivatives have been prepared other than the glycosides or the mesyl and tosyl intermediates previously mentioned. Whitehouse et al. (95) found that the pentaacetyl D-galactosamine diethyl mercaptal in

aqueous acetone and in the presence of mercuric chloride formed the aldehydo sugar. Results of their experiments proved that acyclic amino sugars are detected in natural products by the usual tests made for amino sugars. Hough and Taylor (29) oxidized the diethyl dithioacetal of D-galactose with aqueous peroxypropionic acid and obtained a cyclic disulfone, instead of the expected acyclic unsaturated compound. The disulfone was changed to a D-galactosamine derivative for characterization.

Jeanloz et al. (36) have changed a D-galactosamine derivative into D-idosamine and D-talosamine. Also Jeanloz (35) transformed a 1,6-anhydro-D-galactosamine into a D-gulosamine derivative by the use of an inversion reaction. 3-Deoxy-D-galactosaminic acid hydrochloride has been prepared by Kuhn et al. (51). The 3,4-anhydro-D-galactosamine derivative has been prepared by Gross et al. (21,22) from a D-glucosamine derivative. They prepared benzyl N-carbobenzoxy-3-acetyl-4,6-dimesyl-α-D-glucosamine and found that the 6-mesyl group could be selectively replaced by acetate with an acetic acid-acetic anhydride-potassium acetate reagent. An aqueous potassium hydroxide-dioxane solution removed the acetate group on C-6 and also formed the 3,4-anhydro ring of the D-galactosamine derivative. Reaction of this compound with mesyl chloride produced the 6-mesyl derivative of D-galactosamine, which could

also be formed from the D-glucosamine starting material by the action of sodium isopropylate in isopropyl alcohol-dioxane solution. The selective mesylation of amino sugars thus offers a route to the deoxy- and the polyaminosugars.

Gross et al. (23) have also shown how to separate a product which Heyns and Beck (27) considered to be pure benzyl N-carbobenzoxy-α-D-galactosaminide into two anomers. This investigation proved that glycosidation also formed some of the beta glycoside, although the alpha form predominated. The beta anomer was the more soluble, and separation was possible, by a rather involved procedure making use of the small differences in the solubilities of the two anomers in water and in acetonitrile. Bognar and Nanasi (4) have separated the alpha and beta anomers of N-p-tolyl-D-galactosylamine tetraacetate and other nitrogen derivatives by the use of careful fractional crystallizations. Each of the different methods used to prepare the same compound produced a mixture of anomers. The pure anomers showed a fast mutarotation in hydrochloric acid-methanol solution. In addition, heating of either one of the anomers with acetic anhydride and zinc chloride produced the N-acetylated derivative of the other anomer.

Kochetkov et al. (45) have condensed unprotected amino sugars, including D-galactosamine, with N-carbobenzoxy amino acids in the presence of dicyclohexyl carbo-

diimide in aqueous pyridine to form N-(N'-carbobenzoxy-aminoacyl)-hexosamines. These compounds were converted to N-aminoacyl derivatives with a free amino group by hydrogenolysis in aqueous methanol in the presence of oxalates. The exclusive formation of the nitrogen derivatives seemed to be due to the specific action of the carbodiimide and offers a promising route to the synthesis of a number of natural products.

Bertho and Maier (3) in 1932 laid a foundation for later work by preparing β-azido-tetraacetyl-D-galactose from the alpha chloro compound, and by catalytic hydrogenation of the azide they prepared β-amino-tetraacetyl-D-galactose. Later Heyns and Beck (27) started with benzyl N-carbobenzoxy-α-D-galactosaminide and selectively oxidized the C-6 primary alcohol group to a carboxylic group by using platinum and oxygen. Reduction with palladium and hydrogen then formed the free D-galactosamine uronic acid.

Building on this foundation and by analogy to prior work of Weidmann et al. (90,91,92), Smith et al, (78) in 1965 started with benzyl N-carbobenzoxy-α-D-galactosaminide uronic acid and formed the methyl ester with diazomethane. Reaction with ammonia prepared the amide, acetic anhydride formed the 3,4-diacetyl compound, and triphenylphosphine formed the nitrile. Hydrolysis and catalytic hydrogenation were used to transform the nitrile to the dihydrochloride

of 2,6-diamino-2,6-dideoxy-D-galactose. This work opened the way for a study of the derivatives of diaminogalactose, which will not be included in this review.

Kuhn et al. (46) has synthesized all eight of the 2-amino-hexoses from the pentoses. By the reaction of an aldopentose with aniline or a benzylamine and hydrocyanic acid, they prepared the two epimers of an arylaminonitrile. A study of the amount of each epimer formed indicated, with each pair, that the preponderant one formed led to the hexosamine with the 2-benzylamino or 2-anilino and the 3-hydroxyl groups in the trans position in the Fischer projection formula. For example, from D-lyxose the yield was 23% D-talosamine derivative and 58% D-galactosamine derivative. In a later paper by the same authors (50) a mechanism for the rearrangement of these arylaminonitriles has been presented. Two intermediate products were isolated; the first an iminolactone, while the second was an ene-diaminolactone. The loss of water formed an end product, from which they obtained a 2-amino-3-deoxy-sugar and also a 3-deoxy-2-keto-sugar. A number of derivatives of the 1,4-lactonic acid of D-galactosamine are reported in the same paper. In a third communication (47) the mechanism for the epimerization of the N-substituted-2-amino-2-deoxy-hexanoic acid nitriles by heating in alcohol is explained. It was found to proceed by the reversible cleavage of hydrogen

cyanide to form a Schiff base.

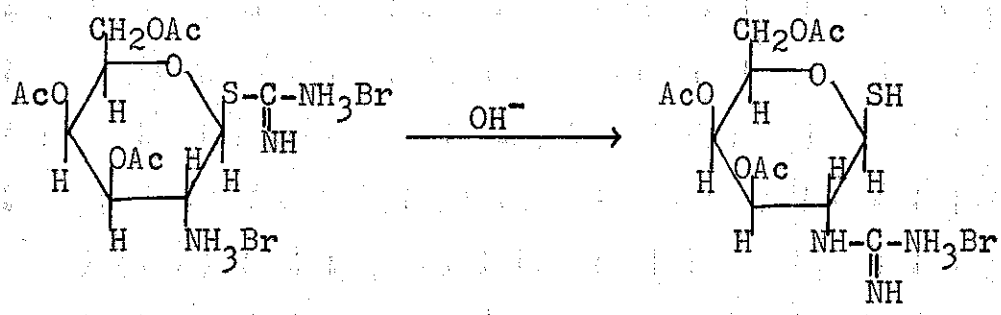
The remainder of the published material on D-galactosamine is concerned with its biochemical aspects--the formation, isolation, or synthesis of derivatives that occur in the tissues of living things. A number of investigations have been made since Kent and Whitehouse's extensive review in 1955 (43). Two studies have been made of methods to separate D-glucosamine and D-galactosamine derivatives as they occur in human blood groups. Annison et al. (1) formed the N-2,4-dinitrophenyl derivative of the amino sugars, then used a boric acid complex of D-galactosamine as a basis for a chromatographic method of separation. Leskowitz and Kabat (58) went one step further and reduced the aldehyde group of the N-2,4-dinitrophenyl derivatives with sodium borohydride to a hydroxy-methyl group. These aminohexitols were separated from other blood group substances by a silicic acid column with 10% ethanol-chloroform. The two amino sugar derivatives were then separated from each other by using a borate-buffered column of silicic acid-celite with 20% ethanol-chloroform. This method was used for samples as small as 0.5 mg. Crimmin (8) has also used sodium borohydride to reduce N-acetyl-α-D-galactosamine to N-acetyl-D-galactosaminol, a reference compound for structural studies of derivatives from blood group mucopolysaccharides.

Marbet and Winterstein (64) have reported a blood-coagulating inhibitor containing D-galactosamine, which they designated as β-heparin. α-heparin is known to contain D-glucosamine. Another derivative of D-galactosamine has been isolated by Crumpton and Davies (9,10) from some strains of Chromobacterium violaceum. This new amino sugar was designated D-fucosamine and its structure was found to correspond to 2-amino-2,6-dideoxy-D-galactose. It was isolated as a crystalline hydrochloride and characterized by forming a number of derivatives.

Tarasiejska and Jeanloz (85) isolated a new amino sugar from streptothricin and streptolin B, and the structure of D-gulosamine was proposed for it. A synthetic material, which agreed with the natural product, was made from methyl N-acetyl-4,6-O-benzylidene-α-D-galactosaminide by using an inversion reaction at C-3 through the mesyl derivative. Lloyd (63) has made a series of C-6 sulfate esters of the various hexosamines, by treating the 1,2,3,4-tetraacetyl-6-trityl compound with chlorosulfonic acid, for comparison with the properties of compounds from the enzymatic degradation of natural aminopolysaccharide sulfates. It was shown that the N-acetyl-6-phospho-D-glucosamine and the N-acetyl-6-sulfo-D-glucosamine both gave a 100% color reaction in the Elson-Morgan reaction, while the corresponding C-3 derivative gave more coloration and the C-4

substituent suppressed the color.

Wolfrom et al. (97) have studied derivatives of D-galactosamine in an attempt to reduce the toxicity of 2-(2-aminoethyl)-2-thiopseudourea, one of the most effective known agents for the protection of biological systems against ionizing radiation. The acetobromogalactosamine hydrobromide was prepared from D-galactosamine hydrochloride by direct reaction with acetyl bromide (96). Reaction of this bromide with thiourea formed a sulfur derivative, which rearranged in basic solution as shown below:



A number of investigators have reported on the enzymatic formation of D-galactosamine derivatives. One of the first of these was Chou and Soodak's (7) proof that pigeon liver extract would cause the acetylation of D-galactosamine. Cardini and Leloir (5) found that an extract, from a yeast enzyme adapted to galactose, would catalyze the phosphorylation of D-galactose and D-galactosamine to an equal extent. They also reported a liver enzyme would

catalyze the transfer of phosphorus from adenosine triphosphate to form a D-galactosamine-1-phosphate. The hydrolysis of the D-galactosamine ester was also much slower than the D-galactose ester. In a later paper by the same authors (6) the N-acetyl-D-galactosamine-1-phosphate was formed by rat liver catalysis. This system was activated by cysteine or magnesium ions, but no effect was noticed with borate, fluoride, acetyl salicylate, penicillin, cortisone, or hydrocortisone. Ethylenediaminetetraacetic acid caused 50% inhibition, while nickel or cobalt ions, instead of magnesium ions, caused 100% inhibition. A mechanism was proposed for the formation of uridine diphosphate acetylgalactosamine in the liver.

Distler et al. (12) prepared D-galactosamine-6-phosphate from D-galactosamine hydrochloride and polyphosphoric acid and found it identical in X-ray powder diffraction pattern and infrared absorption spectra to the product from adenosine triphosphate. The product was acetylated by fungal, bacterial, or mammalian tissue extracts as effectively as D-glucosamine-6-phosphate, although it was not determined whether the same enzyme formed both products.

Kim and Davidson (44) recently synthesized D-galactosamine-1-phosphate. An attempt to prepare this compound via a 1-bromo-D-galactosamine was not successful.

However, a reaction of pentaacetyl-D-galactosamine with anhydrous phosphoric acid yielded a crude product which could be purified by column chromatography. In 1963 Davidson and Wheat (11) prepared the same compound by a combination of chemical and enzymatic procedures. They used a growth of Saccharomyces fragilis on lactose to remove the glucose part of the sugar and cause adaptation of the yeast to D-galactose. The galactokinase formed was extracted and purified, then used for the catalytic synthesis of D-galactosamine-α-1-phosphate. This compound was converted to its N-acetyl derivative and condensed with uridine-5'-monophosphate to yield uridine-diphospho-N-acetyl-D-galactosamine.

This chapter summarizes all the known literature referring to D-galactosamine. It has been given to review the current knowledge about this natural product, and to point out some of the gaps in our knowledge, part of which are filled in by the research reported in this dissertation.

CHAPTER II

DISCUSSION OF RESULTS

The D-galactosamine hydrochloride used in this research project was isolated from chondroitin sulfate, a polysaccharide obtained from the cartilaginous tissues of animals. The method used was the procedure of Gardell (20), with modifications developed in this laboratory in collecting the effluent from the resin column and in testing for the presence of the D-galactosamine hydrochloride (78,93). Although the procedure still involved a considerable period of time, the resulting product is obtained in improved yield and is of high purity.

The next step in the process was the preparation of N-carbobenzoxy-D-galactosamine from D-galactosamine hydrochloride. Modifications of the procedure published by Heynes and Beck (27) were used to increase the yield to seventy-four per cent. These included carrying out the reaction in the presence of potassium bicarbonate, which is more soluble at the lower temperature, instead of sodium bicarbonate. Also, the addition of the carbobenzoxy chloride reagent to the reaction mixture was done in portions over two hours rather than one, while the flask was shaken at zero degrees, instead of at room temperature. This increase of yield in one of the beginning steps of the

synthesis had a significant favorable effect on the time and expense involved in the preparation of the remaining derivatives.

Moreover, all the mother liquors from the preparation and recrystallization processes were collected and evaporated under vacuum until the inorganic salts started to crystallize (23). Sufficient water was added to just dissolve these salts, and the resulting solution was shaken with tetrahydrofuran. The organic material separated into the tetrahydrofuran phase, which was then evaporated; the residue was taken up in water and evaporated again, then hydrolyzed in four normal hydrochloric acid. The resulting solution was decolorized, evaporated to dryness under vacuum, and dissolved in the minimum necessary amount of water. Addition of acetone to incipient turbidity gave, upon standing, crystals of the remainder of the original D-galactosamine. Thus it was possible to obtain N-carbobenzoxy-D-galactosamine with rather small overall losses. In view of the relatively high cost of D-galactosamine hydrochloride this improvement was of great importance.

Since the benzoyl esters of D-galactosamine had not been previously investigated, the N-carbobenzoxy-D-galactosamine (II) was converted by perbenzoylation, with benzoyl chloride in pyridine, into a sirupy mixture of the alpha and beta anomers of 1,3,4,6-tetra-O-benzoyl-N-carbo-

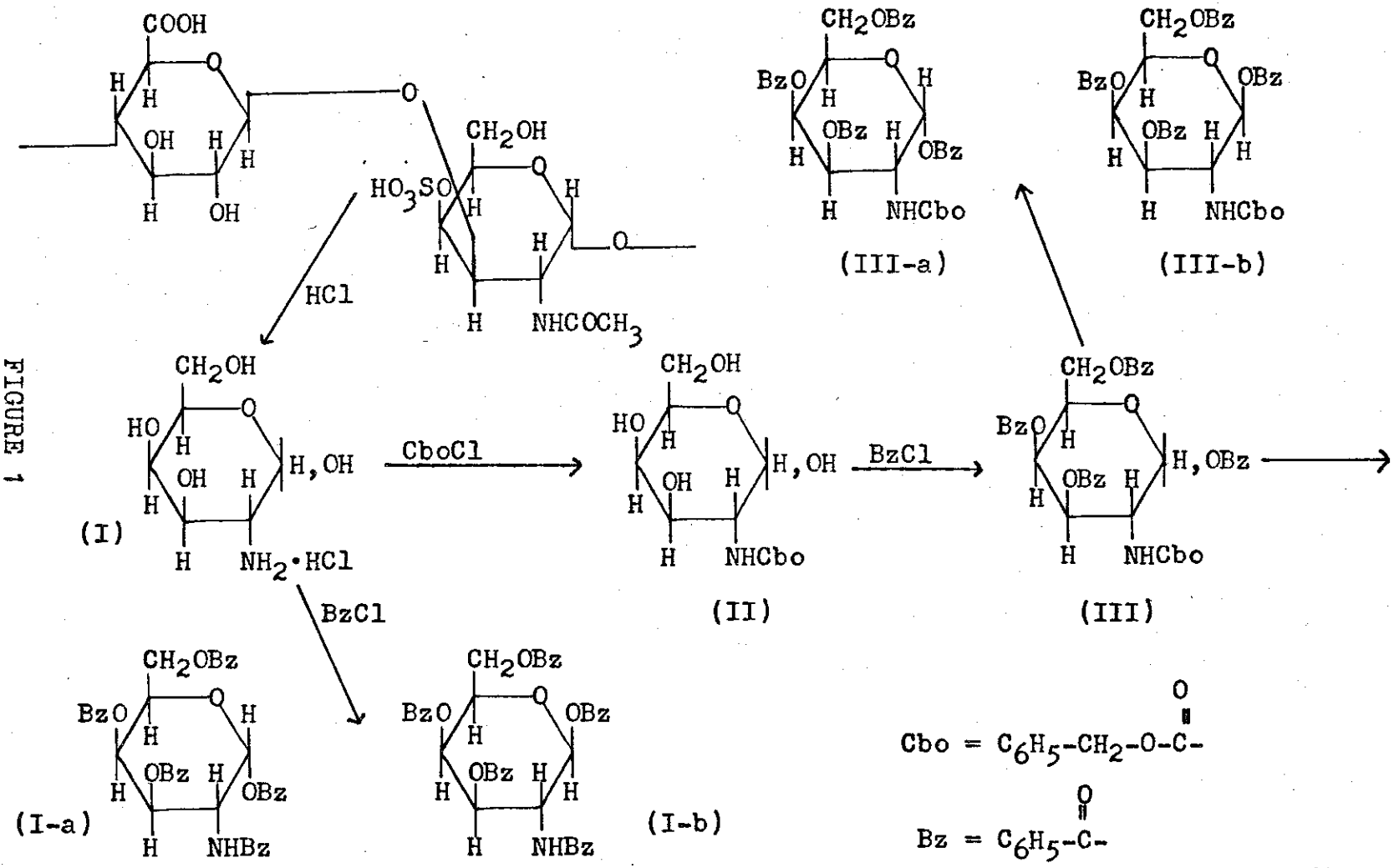

HCl
CboCl
BzCl
BzCl
(I)
(II)
(III)
(III-a)
(III-b)
(I-a)
(I-b)
Cbo = C6H5-CH2-O-C-
Bz = C6H5-C-

FIGURE 1

benzoxy-D-galactosamine (III). Compared to the corresponding acetates, which have been more frequently used (28,30, 61,86), the benzoates are more sluggish in their reactions, but are considerably more stable (70,87). The stability was an important factor with D-galactosamine compounds for they are generally less stable and more difficult to crystallize than the corresponding D-glucosamine derivatives. Part of the sirup (III) was used later for the separation into the pure anomers and the remainder used to prepare the rest of the derivatives.

In a reaction first used by Fischer (15), the sirupy mixture of anomers (III) was treated with a saturated salution of hydrogen bromide in glacial acetic acid. This reagent substituted a bromine atom for the benzoyl group at the first carbon atom of the sugar and simultaneously cleaved the carbobenzoxy group at carbon atom two (87), into benzyl bromide and carbon dioxide, to form the previously unknown 3,4,6-tri-O-benzoyl-1-bromo-α-D-galactosamine hydrobromide (IV). The 1-bromo compound represented the best compromise between reactivity and stability of the possible halogen compounds. As such, it was stable enough to keep for long periods of time, if refrigerated, and the bromine atom was labile enough to be rather easily replaced by other substituents.

Since the benzoyl ester groups blocked the three,

four, and six positions, and the pyranose ring structure blocked carbon atom five, only positions one and two contained possible reaction sites. The nitrogen atom on the second carbon was sufficiently more reactive than the anomeric carbon, so that substitution could be made selectively between the two positions by controlling the conditions of the reactions. This compound then furnished the starting material for all the other derivatives. For, by the use of a series of stereospecific reactions, each of the alpha and beta anomers of two series of compounds was synthesized in pure form. The study of the properties of each of these forms furnished the information necessary for the later resolving of an anomeric mixture of the products from a solution.

The question might well be asked, What factors led to the production of the alpha 1-bromo compound from a mixture of anomers? This was shown to be true for the corresponding D-glucosamine compound by Weidmann (87), for a similar D-galactosamine compound by Onoders and Komano (71) and also by Wolfrom et al. (96), and for some other sugars by Fletcher et al. (16). The theory for the formation of the thermodynamically more stable alpha anomer has been presented by Hassel and Ottar (25) and applied to the carbohydrates by Pigman (73) and also by Lemieux (57). Haynes and Newth (26) mention two general rules that have

been derived from the theory:

1. In the stable form of a substituted sugar, the halogen atom at C-1 is trans to the C-6 group.

2. In the stable form of a substituted sugar, the halogen atom at C-1 is trans to the substituent at C-3.

Observation of the structural formula of (IV) illustrates that for the alpha anomer of the D-galactosamine derivative both of these rules hold, while for the beta form both are violated.

What about the experimental proof? In the first place, the actual reaction in the laboratory was done in glacial acetic acid. Under acid conditions, anomerization is known to take place, so one should expect either an equilibrium mixture of the anomers, or the production of only the more stable one. The compound is not stable enough by itself to use in obtaining any direct chemical proof*, but the high positive specific optical rotation suggests that it is at least mostly the alpha form. As a check on this fact, one quantity of (IV) was made from the pure beta anomer of 1,3,4,6-tetra-O-benzoyl-N-carbobenzoxy-D-galactosamine (III-b) after the anomers of (III) had been separated. The reaction should be expected to proceed with inversion of configuration at C-1, since the nucleo-

*NMR studies might resolve this question, but the requisite equipment has not been available.

philic substitution of bromide for benzoyl should react by an S_N2 mechanism. Crystals of (IV) that were produced had the same melting point and the same specific optical rotation as those produced from a mixture of anomers, suggesting that both were the alpha form.

The main evidence for the alpha structure of (IV), as used by Onodera et al. (71) and also by Wolfrom et al. (96), was its ready conversion into a series of beta glycosides, in yields varying from 28% to 80%, by reaction with the corresponding pure alcohols, in the presence of the organic base pyridine. This inversion reaction produced compounds which all had a low specific rotation indicating they were the beta form, and were of the expected order of magnitude, when compared to the similar D-glucosamine compounds. Although the yields for these compounds varied considerably, the only product that could be isolated was the beta glycoside. The reaction apparently approached an equilibrium, even with the calculated amount of base present, for a sizeable amount of unreacted starting material was left over in each case, and recycling was used to increase the yield of product.

The 3,4,6-tri-O-benzoyl-1-bromo-α-D-galactosamine hydrobromide (IV) was substituted selectively in the nitrogen position by reaction with carbobenzoxy chloride at 0° to give the unstable 1-bromo-N-carbobenzoxy derivative

(VI), which was not isolated. Immediate treatment of this sirup (VI) with silver benzoate, with the exclusion of light and moisture, led by a stereospecific inversion reaction to the formation of 1,3,4,6-tetra-O-benzoyl-N-carbobenzoxy-β-D-galactosamine (VII). This reaction was used and discussed by Weidmann and Zimmerman (88) and also used by Micheel and Köchling (66) for a reaction with a similar D-glucosamine derivative.

Treatment of (IV) with benzoyl chloride at 0° was also used to produce another unstable intermediate--3,4,6-tri-O-benzoyl-N-benzoyl-1-bromo-α-D-galactosamine (VIII). All attempts to crystallize this sirup resulted in a rearrangement to structure (X). A reaction of (VIII), as a sirup, with powdered silver benzoate in the absence of light and moisture did not form the expected product--1,3,4,6-tetra-O-benzoyl-N-benzoyl-β-D-galactosamine (IX). In one case crystals were obtained that did not correspond to the expected analysis. In a later case, a thin-layer chromatogram on a fresh quantity of sirup demonstrated that a number of products were formed, none of which were either the alpha (XI) or the beta (IX) form of the expected product.

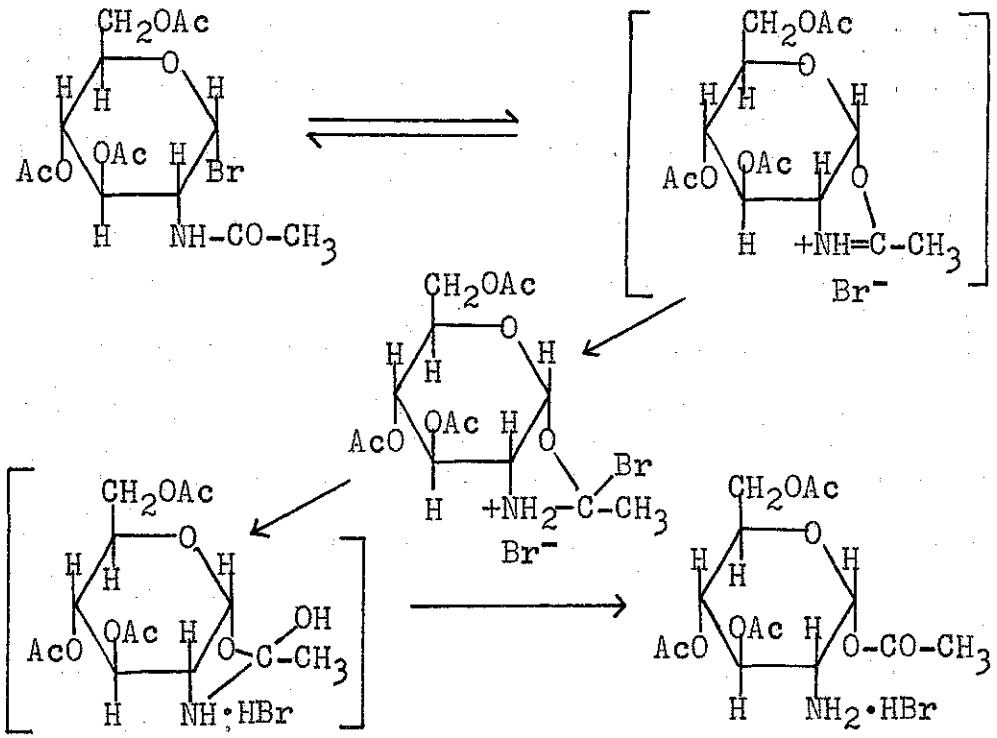
Since this reaction was successfully used to produce (VII) from the same bottle of silver benzoate, one must conclude either that this is not a reliable reaction, or

that the presence of a carbobenzoxy group on the nitrogen atom instead of a benzoyl function, in some way promoted the reaction, perhaps by the stabilization of an intermediate state. However, it must be remembered that this is a two-phase system, for the silver benzoate does not dissolve in the chloroform used as a solvent. As such, the reaction may be similar to many catalytic processes in that the rate could be dependent on such factors as surface activity of the solid, amount of surface area, or the rate of shaking the mixture. At any rate, the same compound as (IX) was separated from the mixture of anomers (I-b) and carefully purified by chromatographic methods.

As previously mentioned (VIII) could be changed by the presence of water into the migration product, (X), 1,3,4,6-tetra-O-benzoyl-α-D-galactosamine hydrobromide. The reaction was carried out by mixing a chloroform solution of the sirup with an aqueous solution of diethyl ether and letting the mixture stand at room temperature. White crystals started to form in a few minutes. However, the yield (for two steps) could not be made to approach that reported for the D-glucosamine compound, and the product was not analytically pure when precipitated, as was also reported for D-glucosamine. In addition, analysis indicated that it crystallized as a monohydrate. An equilibrium seemed to be reached, for additional crops

of progressively less pure crystals could be removed over a period of several months. Heating with reflux in diethyl ether or in tetrahydrofuran made no apparent difference in the amount of reaction. The addition of a weak acid, p-toluene sulfonic acid, also had little, if any, effect, but the reaction may be subject to other catalysts.

Although the mechanism of the reaction for the D-galactosamine derivative has not been studied, an intermediate for the reaction of a 1-bromo-N-acetyl derivative of D-glucosamine was isolated by Micheel and Petersen (67). Kulkarni and Zimmerman (52) have also studied the intermediates in other derivatives of the same series. Micheel and Petersen (67) proposed the following mechanism for the migration:



The migration of substituents in sugars with free hydroxyl groups has been known for many years. In 1920 Fischer (14) found an acyl group migration in some of the glycerides, while the same reaction was first observed in the amino sugars by White (94). In the case of sugars, it is known that the groups on carbon atoms one and two must be cis (carbon atom one, alpha for D-glucosamine, D-galactosamine, etc.) to each other for the reaction to occur. Therefore, this reaction is stereospecific and may be used, as in this investigation, for the production of the pure alpha anomer.

This migration product (X) was treated with benzoyl-chloride to form 1,3,4,6-tetra-O-benzoyl-N-benzoyl-α-D-galactosamine (XI), which was subsequently proven identical to a product isolated from a mixture of anomers (I-a). Alternately, a reaction of (X) with carbobenzoxy chloride was used to produce 1,3,4,6-tetra-O-benzoyl-N-carbobenzoxy-α-D-galactosamine (XII), a product which was proven identical to (III-a).

Before a discussion of the separation of the mixture of anomers, an explanation of the chromatographic methods used in their isolation should be given. A large part of the success in isolating the pure anomers in the two series of compounds must be credited to the discovery of a suitable solvent system for the differential migration of the

alpha and beta anomers by thin-layer chromatography. After a number of experiments, it was found that commercial chloroform, which contains five-tenths of one per cent ethanol, when used with glass plates coated with silica gel, caused the alpha form to move considerably faster than the beta in an ascending chromatogram. The silica gel was mixed with a phosphor before coating, so that detection of the spots could be made with an ultraviolet light. This detection method would work only with sugar derivatives containing enough benzene rings in the molecule to absorb the ultraviolet light rays.

After its development, this method furnished a rapid, accurate way of detecting the anomeric purity of a chloroform solution of any sirup before its crystallization was attempted. The method, of course, is not limited to D-galactosamine derivatives, but could profitably be used with any of the amino sugars. Its use on some of the previously reported series might conceivably show that some contamination of the anomers was present. Moreover, the results of thin-layer chromatography were readily transferred to the column form. Silicic acid plus a phosphor, when activated by heating at 125° for one-half hour, would separate a sirup into its anomers upon elution with commercial chloroform. Ultraviolet light was used to detect the elution progress of the anomers in a quartz glass column

about one meter in length. Alternately, the effluent was separated by an automatic fraction collector and each fraction analyzed by thin-layer chromatography. One of the typical chromatograms obtained is shown in figure 3. In view of the very similar properties of the two anomers of any sugar series, and of the practical difficulties of complete separation by the process of fractional crystallization, this method has tremendous advantages. There are, however, practical difficulties in trying to separate large amounts of material by column chromatography.

As described in the experimental details, a mixture of the alpha and beta anomers for each of the two series of compounds was prepared by a non-stereospecific reaction. Then in each case the least soluble anomer was induced to crystallize first. After its removal the second anomer was separated. In the 1,3,4,6-tetra-O-benzoyl-N-benzoyl-D-galactosamine series the alpha anomer was present in larger amount and crystallized first. The other series--1,3,4,6-tetra-O-benzoyl-N-carbobenzoxy-D-galactosamine--led to the beta anomer from the first crystals. In both cases the second anomer was very difficult to obtain.

Extensive attempts to crystallize the alpha anomer in the N-carbobenzoxy series, as described in the experimental details, were not successful. The same product was obtained both by a stereospecific chemical reaction and by

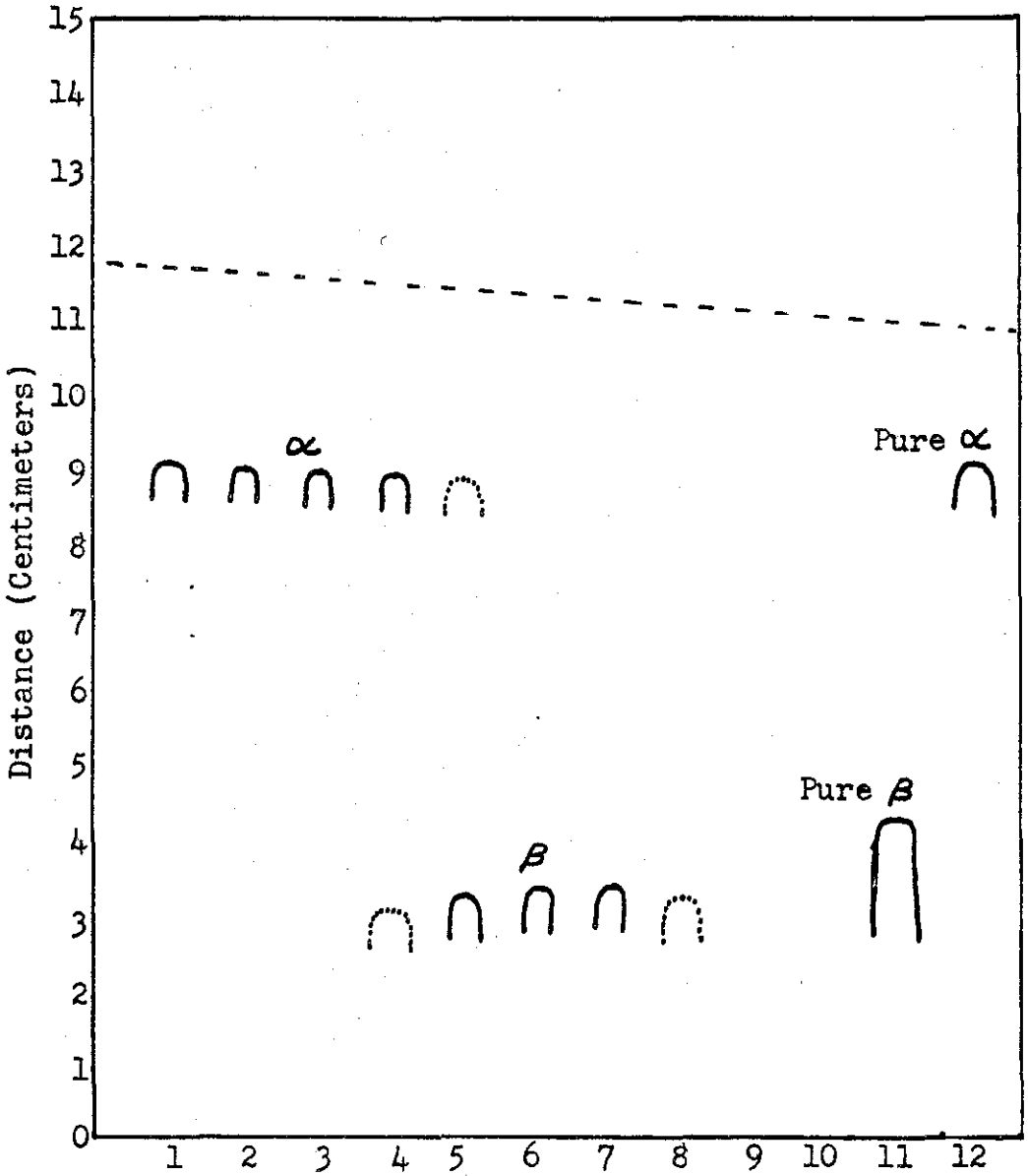


FIGURE 3

SEPARATION OF THE ANOMERS OF 1,3,4,6-TETRA-O-BENZOYL-N-BENZOYL-D-GALACTOSAMINE WITH A SILICA GEL COLUMN AND AUTOMATIC FRACTION COLLECTOR

separation of the mixture, but neither one has been crystallized at this time. The mixture of the N-carbobenzoxy anomers had a very small amount of the alpha form present, which made the attempt to crystallize it more difficult.

In an effort to explain the small amount of the alpha form present, a limited rate investigation was made of the benzoylation of N-carbobenzoxy-D-galactosamine. The reaction was run in the usual way with benzoyl chloride in pyridine at 0^{o}, except that at timed intervals an aliquot of the reaction mixture was withdrawn. Each of these samples were put into cold water, and the benzoylated sugar extracted with chloroform, which was then washed, dried, and finally concentrated by vacuum distillation. Each of these fractions were then analyzed by a thin-layer chromatogram to determine qualitatively the relative amounts of each anomer present. In addition, each sample was diluted to exactly five millliliters with chloroform, and the optical rotation was determined for each sample. Aliquots were removed at various times over a period of twenty-four hours. Toward the end of the run some brown color appeared in the solution indicating some degradation of the product, which rendered the last few optical rotations less reliable.

However, both methods of detection showed that the beta anomer was formed first, which should be expected due

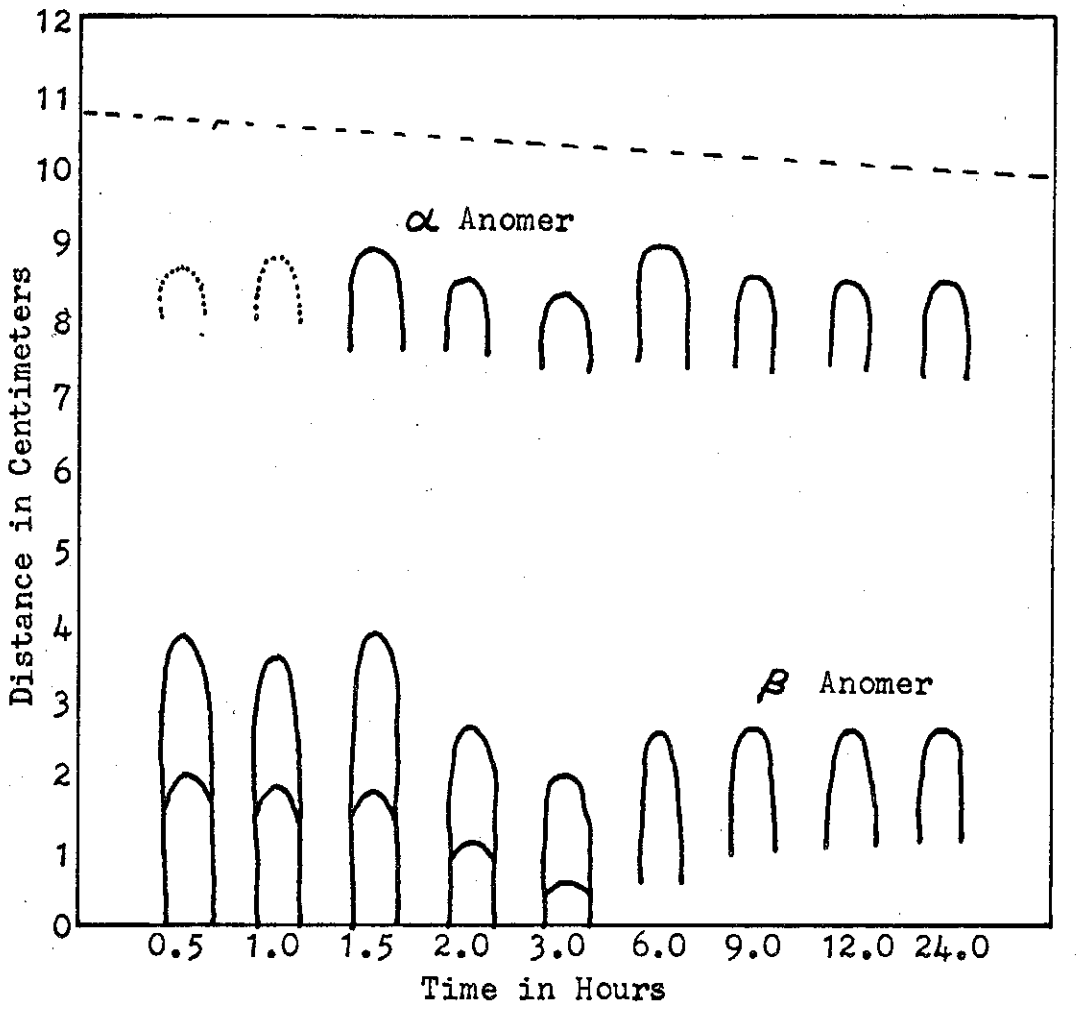


FIGURE 4

SEPARATION OF ALPHA AND BETA ANOMERS IN ALIQUOTS FROM BENZOYLATION OF N-CARBOBENZOXY-D-GALACTOSAMINE

to its more favorable equatorial position. This anomer was later partially converted to the alpha form, with equilibrium being reached after about six hours of reaction time. This, when compared to the usual reaction time of three hours, explained the small percentage of the alpha anomer present. It also illustrated the slower reaction rate of the D-galactosamine compounds, compared to the similar compounds in the D-glucosamine series. In addition, it is of particular interest that this anomerization of the 1-benzoate seems to be in accord with the anomerization of benzyl N-carbobenzoxy-D-glucosaminides previously reported by other workers from this laboratory (24,84), suggesting that anomeric equilibrations may well constitute a process of general importance among amino sugar derivatives, the significance of which has not been appreciated heretofore.

CHAPTER III

PREPARATION AND SEPARATION OF THE ANOMERS

D-Galactosamine Hydrochloride (I). According to the method of Wheat and Davidson (93), 250 g of chondroitin sulfate were dissolved in water and stirred with Dowex 50W-X-8 ion exchange resin. After one hour the pasty mass was filtered with suction and washed with water. Barium hydroxide solution was used to adjust the pH to 6.0, then an equal volume of concentrated HCl was added and the mixture heated under reflux overnight. The resulting solution was decolorized with carbon, filtered, and vacuum distilled to concentrate the product. Distillation from alcohol and then benzene served to remove the final traces of acid. Addition of acetone to the alcohol solution caused white crystals to form. These were dissolved in a solution of equal parts of methanol and water and the yellow solution decolorized with carbon.

Following the method of Gardell (20), the filtrate was run through a column 5 cm in diameter and 1 meter long packed with Dowex 50W-X-8 ion exchange resin. As shown by Smith et al. (78), the contaminents consist of a small amount of D-glucosamine with larger amounts of NH_4Cl. The column was prepared by shaking the resin with 2 N HCl, then rinsing with distilled water until the effluent became

neutral. The solution was then allowed to flow through the column at the rate of 30 ml per hour. A water rinse was used to remove hydrogen ions and inorganic salts. Elution with 0.2 N HCl removed the adsorbed nitrogen compounds in the order of increasing basicity. This results in D-glucosamine hydrochloride coming through first, followed by D-galactosamine hydrochloride, and finally by the ammonium chloride solution. The fractions collected can be analyzed by comparison of color in the Elson-Morgan test, by specific optical rotation, by specific resistance, or by X-ray powder photographs made on residual solids. Specific rotations were used for this work. White crystals were obtained by evaporating the solvent in vacuo, dissolving the sirup in absolute alcohol, and adding acetone to turbidity. Long needle-like crystals formed after cooling overnight. Yield 32.4 grams. $(\alpha)_D^{25} = +93^\circ$ (c = 2.10% in water).

N-Carbobenzoxy-D-Galactosamine (II). A mixture of 10.8 g of D-galactosamine hydrochloride (I), 11.3 g of potassium bicarbonate, and 75 ml of distilled water was cooled to 0° with shaking. After the solids were dissolved, 10.8 g of carbobenzoxy chloride were added at the rate of 3 ml every ten minutes with continued shaking. The shaking was continued for a total period of two hours. Then 100 ml

of ethyl acetate were added to the white pasty mass, and the mixture was heated in the water bath until dissolution was complete, as indicated by two clear layers. This was stored overnight in the refrigerator and the crystalline product was filtered, washed with ethyl acetate, dissolved in a minimum amount of hot methanol, and ethyl acetate added to turbidity. Slow cooling produced white crystals, which were filtered and washed successively with ethyl acetate, ether, and petroleum ether to give 11.5 g (74%) melting at 177-78°.

1,3,4,6-Tetra-O-Benzoyl-N-Carbobenzoxy-α,β-D-Galactosamine (III). Five grams of (II) dissolved in 60 ml of pyridine were cooled in an ice bath and 15 ml of benzoyl chloride were added dropwise, while the mixture was stirred. A white precipitate began to form after about one-half of the benzoyl chloride had been added, and the liquid phase assumed a pink color. After standing overnight in the cold, the dark red liquid containing the white precipitate was poured into a mixture of 51 ml concentrated HCl, with equal volumes of ice water and chloroform, and the combined organic extracts were washed with cold 1 N HCl, then twice with ice water, and dried at 0° over anhydrous sodium sulfate. The filtrate was concentrated in vacuo, and then re-evaporated twice from toluene to give a thick, yellow sirup. One quantity of the sirup was used for the separa-

tion of the anomers and the others were changed into (IV).

3,4,6-Tri-O-Benzoyl-1-Bromo-α-D-Galactosamine Hydrobromide (IV). The sirup, (III), was treated with about four to five volumes of saturated HBr/ glacial acetic acid in the same flask, and, after thorough shaking, it was stored in the cold. The viscid, partially crystalline mixture was then concentrated in vacuo and, when nearly dry, ether was added. Additional crystals formed upon cooling. These white crystals were washed thoroughly with ether and dried in vacuo over KOH/CaO. The crude product was recrystallized by dissolution in cold tetrahydrofuran, followed by addition of ether to turbidity to give yields varying between 74% and 81% for the two steps (different trials). The product melted at 163-64° (decomp.) and was used as starting material for the preparation of the other derivatives. $(\alpha)_D^{32} = +139°$ (c = 1.19% in tetrahydrofuran).

$C_{27}H_{24}NO_7Br \cdot HBr$ (635.32).

Calculated:	51.05% C	3.97% H	2.21% N
Found:	51.41% C	4.10% H	2.18% N

3,4,6-Tri-O-Benzoyl-1-Bromo-N-Carbobenzoxy-α-D-Galactosamine (VI). A mixture of 1.27 g of (IV) suspended in 25 ml of absolute chloroform and 1.0 g of sodium bicarbonate dissolved in 20 ml of water was cooled in an ice bath. After addition of 0.40 ml of carbobenzoxy chloride, the

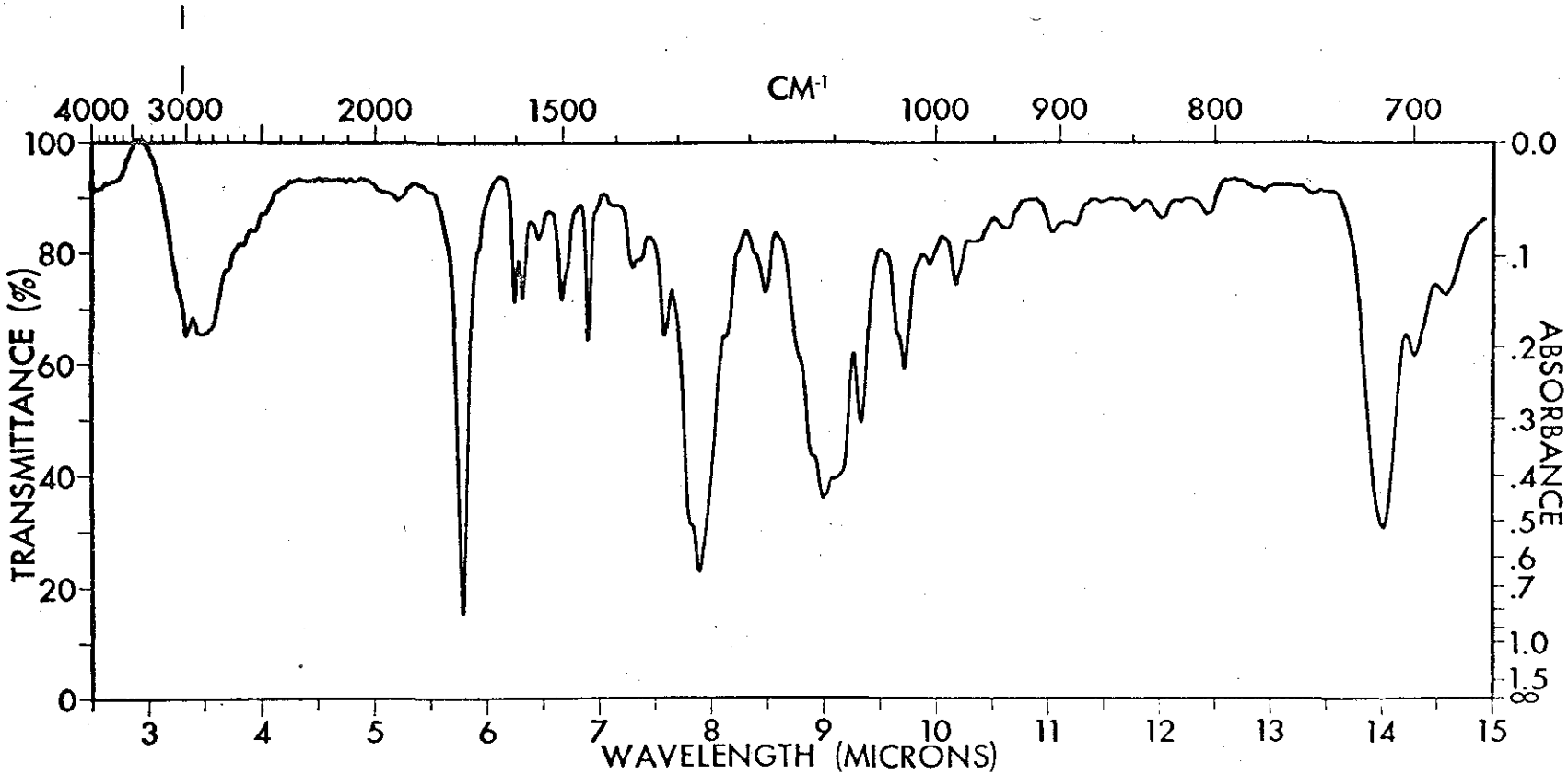


FIGURE 7

INFRARED SPECTRUM OF 3,4,6-TRI-O-BENZOYL-1-BROMO-α-D-GALACTOSAMINE HYDROBROMIDE (IV)

mixture was shaken in ice water for three hours. The chloroform layer was separated, washed with saturated ice-cold sodium bicarbonate solution, then twice with ice water, and dried over anhydrous magnesium sulfate at 0°. The chloroform filtrate was concentrated to a colorless sirup on the evaporator. This water-sensitive compound was too unstable to be crystallized and was used directly in the following step.

1,3,4,6-Tetra-O-Benzoyl-N-Carbobenzoxy-β-D-Galactosamine (VII) and (III-b).

a. Some fresh anhydrous magnesium sulfate was added to the chloroform solution from the preceding step, (VI), and 2.0 g of powdered silver benzoate were added. The flask was covered with black rubber, vented through a $CaCl_2$ drying tube, and shaken at room temperature for 48 hours. Solids were then filtered out, and the filtrate extracted twice with ice-cold 2% aqueous ammonia and twice with ice water to remove traces of silver salts. After addition of magnesium sulfate the solution was stored overnight at 0°. Concentration of the filtrate and addition of hexane produced oily drops, which crystallized with scratching of the flask. Crystals with a slight yellow color in a crude yield of 70% (2 steps) were produced. These were recrystallized four times from hot toluene-diisopropyl ether

to give 0.28 g (19.2%, 2 steps) of fluffy white crystals. m.p. = 167-68° $(\alpha)_D^{23}$ = +21.2° (c = 1.04% in pyridine).

b. Separation From The Mixture. Addition of a little diethyl ether and an excess of diisopropyl ether to a quantity of the sirup (III), caused oily drops to form, which slowly crystallized when left standing at room temperature. These, when recrystallized from toluene-hexane or toluene-diisopropyl ether, proved to be the beta anomer (III-b). Additional crystals were later obtained by running a chloroform solution of the remaining sirup through a silicic acid column, in which the alpha form moved the more rapidly. Total yield of beta anomer was 0.34 g (11.7%, 2 steps). m.p. = 167-68° $(\alpha)_D^{23}$ = +22.6° (c = 0.97% in pyridine). (VII) and (III-b) were proven identical by the use of melting point, specific rotation, mixed melting point, infrared spectra, and thin-layer chromatography.

$C_{42}H_{35}NO_{11}$ (729.75)

Calculated:	69.14% C	4.83% H	1.92% N	24.12% O
Found:	69.13% C	4.93% H	2.24% N	24.04% O

3,4,6-Tri-O-Benzoyl-N-Benzoyl-1-Bromo-α-D-Galactosamine (VIII). This compound was prepared from (IV) by the same directions already given for the preparation of (VI), except that benzoyl chloride was used as the reagent instead of carbobenzoxy chloride. The product was also

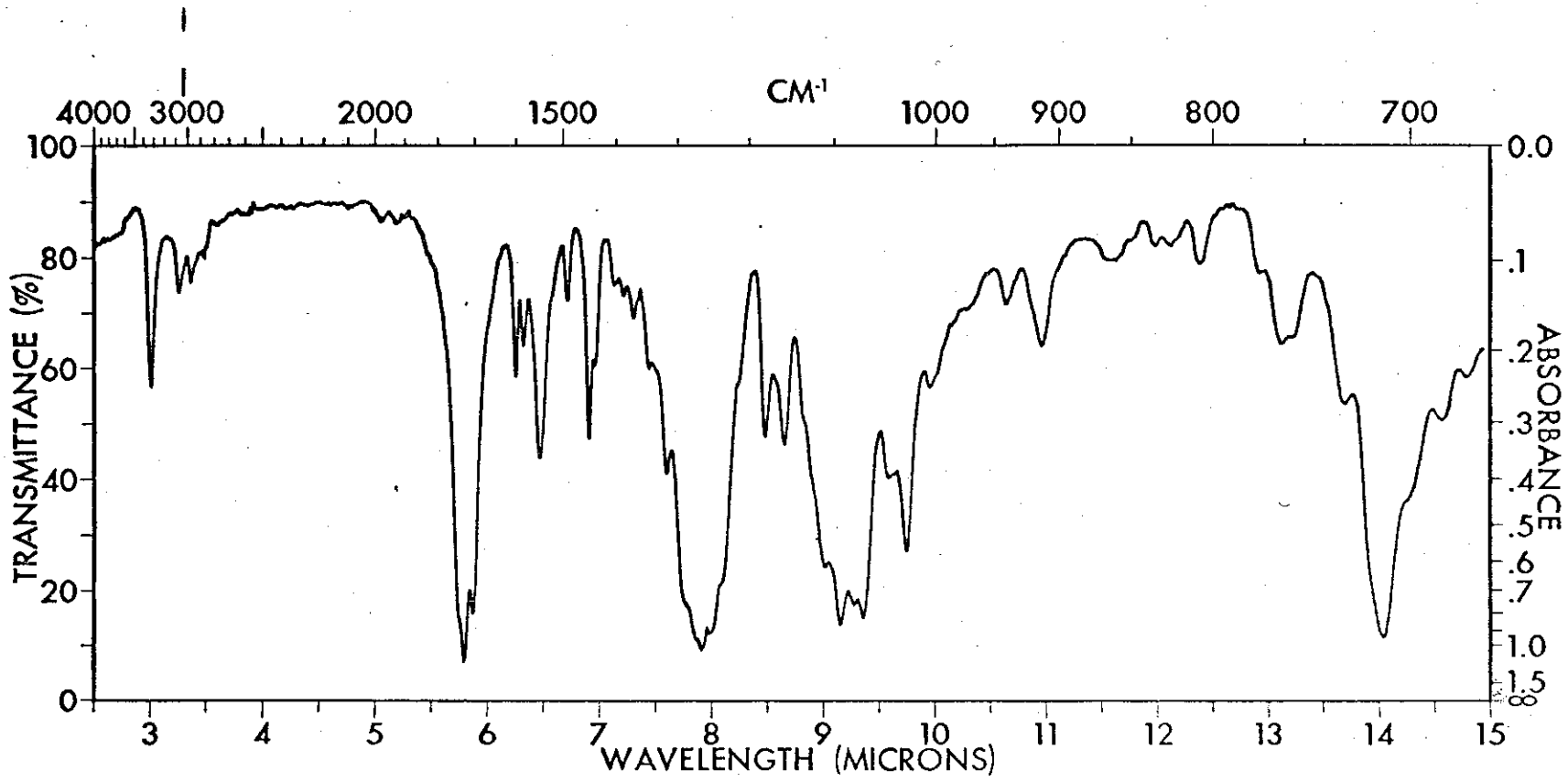


FIGURE 8

INFRARED SPECTRUM OF 1,3,4,6-TETRA-O-BENZOYL-N-CARBOBENZOXY-β-D-GALACTOSAMINE (VII)

extracted and dried by the same procedure. The unstable sirup was left in the chloroform solution and used directly in the following step.

1,3,4,6-Tetra-O-Benzoyl-N-Benzoyl-β-D-Galactosamine (IX) and (I-b).

a. (IX). This compound was prepared from the sirup (VIII) by the same directions given for the preparation of (VII), except that the final sirup was analyzed by thin-layer chromatography before attempting to crystallize it. Results indicated that a mixture of products was obtained by the reaction with the silver benzoate, none of which were the same as the pure beta anomer (I-b). Further identification was not attempted. It should be noted, however, that a similar reaction was successfully used to produce (VII).

b. (I-b) from separation of the mixture. The mother liquor from the alpha anomer was concentrated, fresh diisopropyl ether was added in excess, and the solution was stored in the freezer for several days. Decanting the liquid left a yellow gum, which was dissolved in toluene. Crystals formed when diisopropyl ether was added. These were mostly the beta anomer contaminated with a small amount of the alpha form. The decanted liquid also produced a further crop of a mixture of crystals when left standing at room temperature. The pure beta anomer was obtained in

each of the following ways:

1. Repeated fractional crystallizations from toluene-diisopropyl ether in which the alpha form is the less soluble.

2. Running a chloroform solution of the sirup through a 100 cm column of silicic acid containing a phosphor. A quartz glass column was used, so the progress of the sirup could be followed by ultraviolet light. As described elsewhere in this report, the alpha anomer moved faster through the column than the beta, and pure solutions of each form were obtained which crystallized from diisopropyl ether.

The beta anomer was recrystallized from toluene-diisopropyl ether to give a total yield of 0.36 g (5.2%). m.p. = 163-64°. Thin-layer chromatography was used to prove this was the pure beta anomer.

$(\alpha)_D^{23} = +37.2°$ (c = 1.02% in pyridine). $C_{41}H_{33}NO_{10}$ (699.71).

Calculated:	70.38% C	4.75% H	2.00% N	22.87% O
Found:	70.88% C	4.79% H	2.19% N	22.41% O

1,3,4,6-Tetra-O-Benzoyl-α-D-Galactosamine Hydrobromide (X). The sirup, (VIII), resulting from the reaction of 0.38 g of (IV) was dissolved in the minimum amount of absolute chloroform, 45 ml of diethyl ether, saturated with water, were added, and the solution allowed to stand for twenty-four hours at room temperature. White crystals started to form within a half hour. After cooling, the

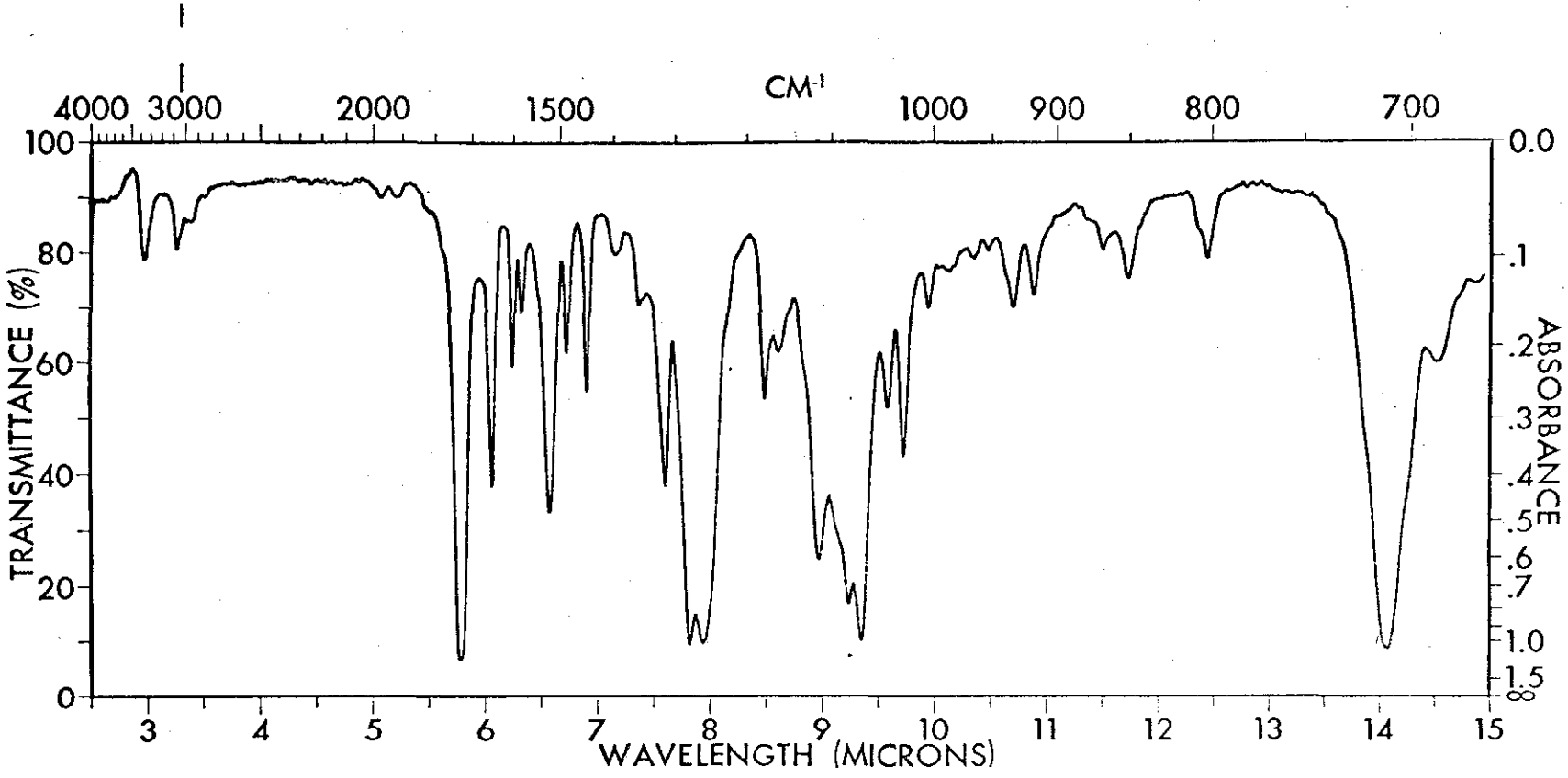


FIGURE 10

INFRARED SPECTRUM OF 1,3,4,6-TETRA-O-BENZOYL-N-BENZOYL-β-D-GALACTOSAMINE (I-b)

solution was filtered, and the hydrated crystals were washed with ether, to yield 0.23 g (56.1%, 2 steps). The product was recrystallized from methanol-diethyl ether. m.p. = 204° (decomp.). $(\alpha)_D^{23} = +96.3°$ (c = 0.572% in methanol).

$C_{34}H_{29}NO_9 \cdot HBr \cdot H_2O$ (694.54).

Calculated:	58.71% C	4.64% H	2.01% N
Found:	58.85% C	5.02% H	2.39% N

1,3,4,6-Tetra-O-Benzoyl-N-Benzoyl-α-D-Galactosamine (XI) and (I-a).

a. (XI). A mixture of 60 mg of (x) in 20 ml absolute chloroform, 1.0 g of sodium bicarbonate dissolved in 20 ml of ice water, and 0.20 ml of benzoyl chloride was shaken for three hours in an ice bath. After separation of the liquid layers, the chloroform phase was washed with ice-cold saturated sodium bicarbonate solution, then twice with ice water, and dried at 0° over anhydrous magnesium sulfate. The filtrate was concentrated in vacuo and diisopropyl ether added to turbidity. White crystals formed on standing. Yield 50 mg (81%). The product was recrystallized from hot toluene-diisopropyl ether. m.p. = 195-96°. $(\alpha)_D^{30} = +130°$ (c = 0.38% in pyridine).

b. (I-a) from separation of the anomers. Two and sixteen-hundredths grams (0.01 M) of (I) were dissolved in 30 ml of pyridine and cooled in an ice bath. Then 6.0 ml

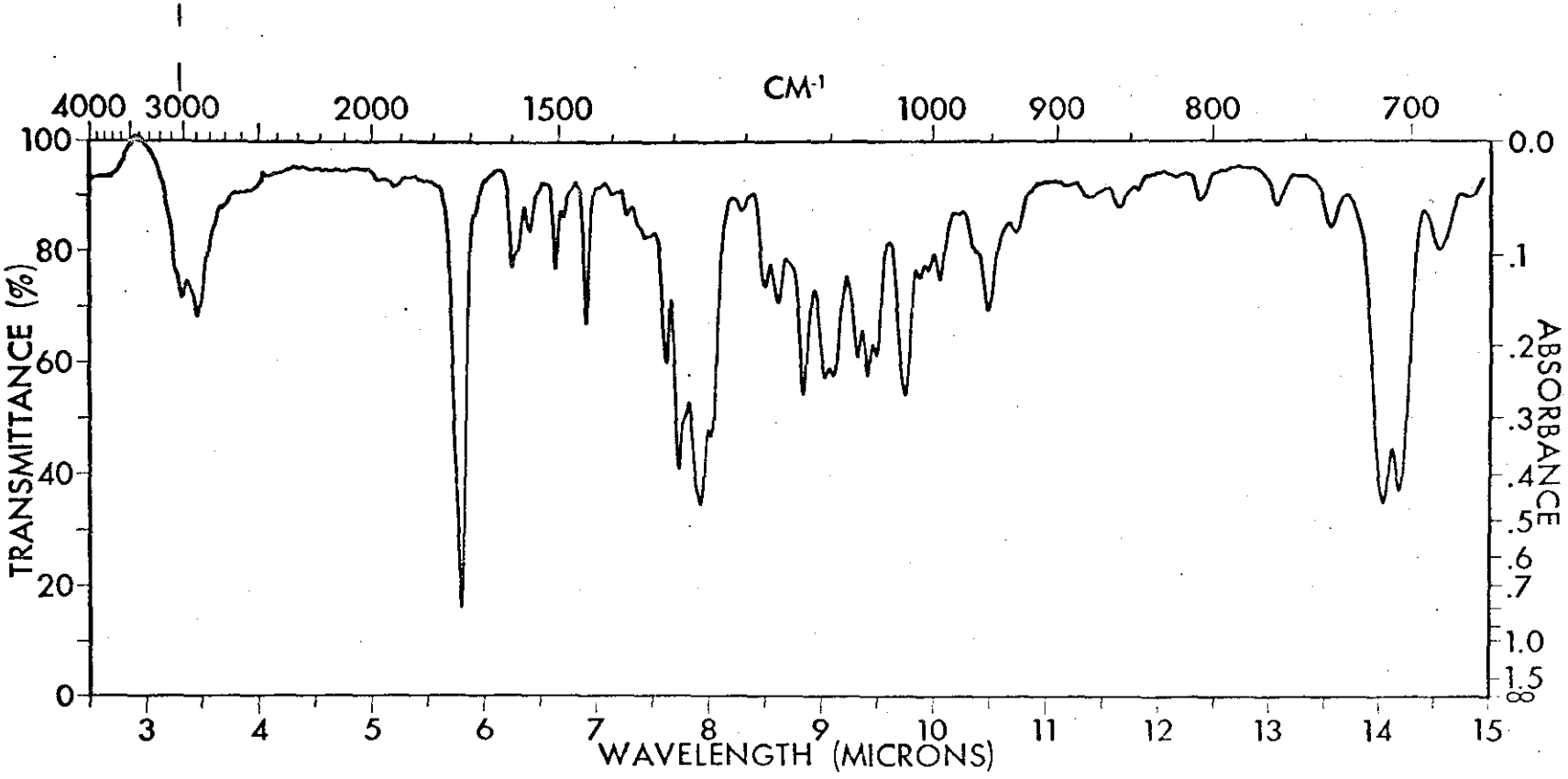


FIGURE 11

INFRARED SPECTRUM OF 1,3,4,6-TETRA-O-BENZOYL-α-D-GALACTOSAMINE HYDROBROMIDE (X)

of benzoyl chloride dissolved in 24 ml of pyridine were added dropwise during the first 30 minutes, while the mixture was being stirred in an ice bath. Stirring in the cold was continued for a total of four hours, after which the red solution was stored in the refrigerator. The sirup was separated by the same procedures used for (III). Addition of a little diethyl ether, an excess of diisopropyl ether, scratching the flask, and standing for several days at room temperature, produced a crop of white crystals. They were recrystallized from hot toluene-diisopropyl ether to give the pure alpha anomer (I-a). Total yield 2.04 g (29.2%). m.p. = 195-96°. $(\alpha)_D^{27} = +133°$ (c = 1.23% in pyridine). Crystals of (XI) and (I-a) were proven identical by mixed melting point, specific rotation, infrared spectra, and thin-layer chromatography.

$C_{41}H_{33}NO_{10}$ (699.71)

Calculated:	70.38% C	4.75% H	2.00% N	22.87% O
Found:	70.56% C	4.62% H	2.06% N	22.81% O

1,3,4,6-Tetra-O-Benzoyl-N-Carbobenzoxy-α-D-Galactosamine (XII) and (III-a).

a. (XII). A mixture of 0.16 g of (X) in 25 ml absolute chloroform, 1.0 g of sodium bicarbonate in 25 ml of distilled water, and 0.20 ml of carbobenzoxy chloride was shaken for four hours in an ice bath. The chloroform

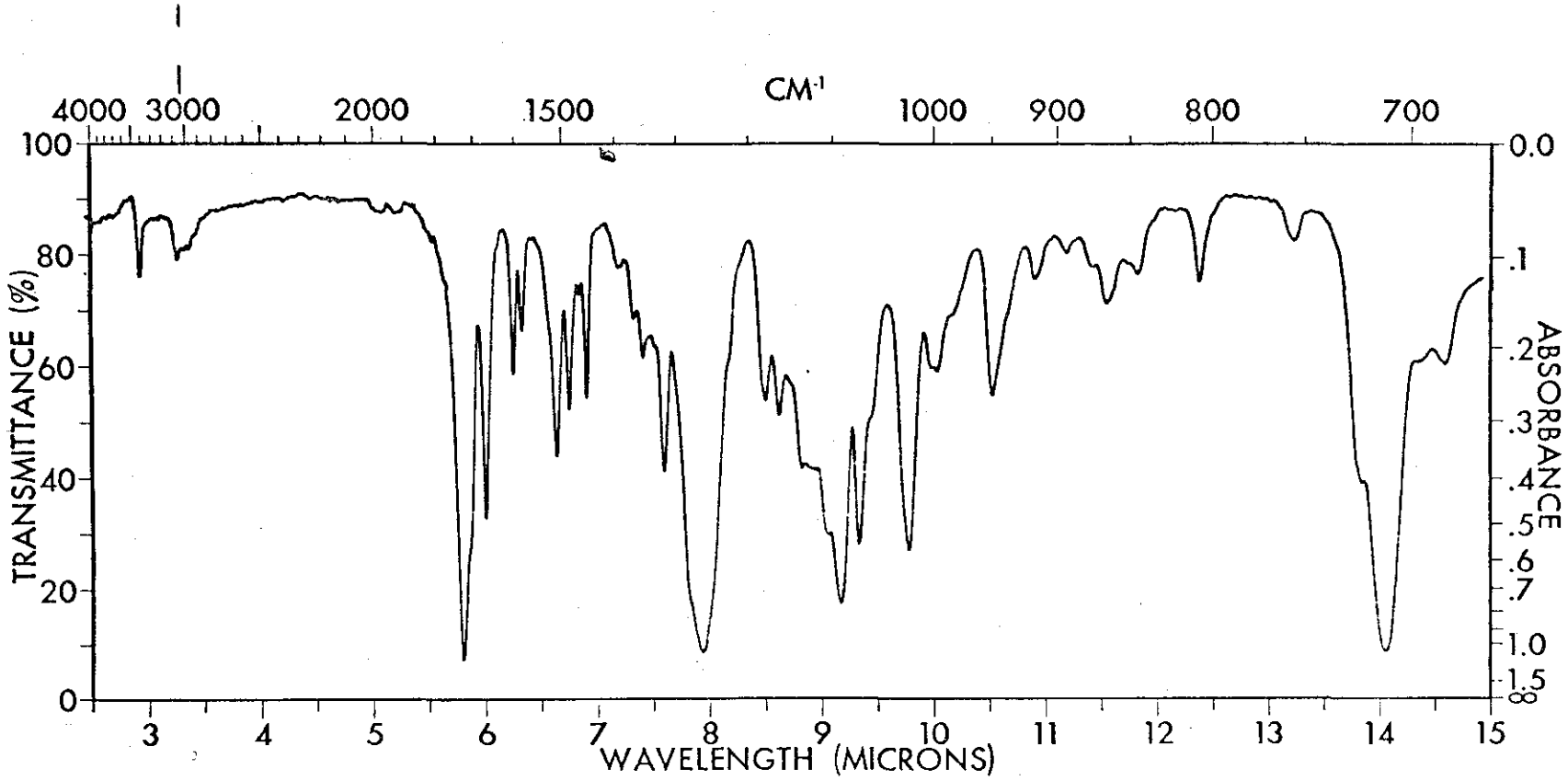


FIGURE 12

INFRARED SPECTRUM OF 1,3,4,6-TETRA-O-BENZOYL-N-BENZOYL-α-D-GALACTOSAMINE (XI)

layer was separated, washed with ice-cold saturated sodium bicarbonate solution, then twice with ice water, and dried at 0° over anhydrous magnesium sulfate. The filtrate was concentrated *in vacuo*. Extensive attempts to crystallize the sirup were not successful. Eight different quantities were prepared, but every combination of solvents used produced an oil or oily flocculent particles that could not be filtered, and which decomposed on standing. Thin-layer chromatography was used to prove that the sirup was a single compound and identical with (III-a).

b. (III-a) from separation of the anomers. The mother liquor remaining from the crystallization of the beta anomer, (III-b), from the sirup (III) was purified by running through a silicic acid column. Extensive attempts to crystallize the small amount of pure alpha sirup, (III-a), obtained were not successful. Thin-layer chromatography was used to prove that it was a single compound and identical with (XII).

Rate of Benzoylation of N-Carbobenzoxy-D-Galactosamine (II). Two and seventy-three hundredths grams of (II) were dissolved in pyridine and diluted to 65 ml. While stirring in an ice bath, 10 ml of benzoyl chloride were added during the first thirty minutes. Two milliliter aliquots were removed at timed intervals. Each sample was

poured into ice water containing 20 ml of chloroform and 1 ml of concentrated hydrochloric acid. The water layer was extracted with two more 20 ml volumes of chloroform. The combined organic phase was washed with 1 N hydrochloric acid, twice with ice water, and dried at 0° over anhydrous sodium sulfate. Each sample was filtered, concentrated, and some used for a thin-layer chromatogram. Then each solution was diluted to 5.0 ml with chloroform and the optical rotation determined. Results are summarized in table I and in figures 4 and 5.

Chromatographic Methods. The chromatographic separations reported in this investigation, both the thin-layer and the column type, used commercial chloroform, which contains 0.5% ethanol, as the solvent system. The thin-layer chromatograms were run on 9 x 9 inch glass plates in glass tanks. Solvent was placed only on the bottom of the container. The adsorbent was silica gel containing a trace of phosphor, with ultraviolet light used as the detector. The column separations were done in a quartz glass column 3 cm in diameter and 70 cm in length, containing silicic acid and phosphor.

TABLE I

CHANGE OF ROTATION WITH TIME IN BENZOYLATION OF N-CARBOBENZOXY-D-GALACTOSAMINE

Sample Number	Total Elapsed Time (Hours)	Rotation Angle
1	0.5	4.54
2	1.0	5.28
3	1.5	5.66
4	2.0	5.64
5	3.0	6.20
6	6.0	6.89
7	9.0	6.49
8	12.0	6.57
9	24.0	7.09

CHAPTER IV

PREPARATION OF THE BETA GLYCOSIDES

Methyl 3,4,6-Tri-O-Benzoyl-β-D-Galactosaminide Hydrobromide (V-a). Ten milliliters of distilled methanol were used to dissolve 0.50 g of 3,4,6-tri-O-benzoyl-1-bromo-α-D-galactosamine hydrobromide (IV), and four drops of pyridine were added. The solution was left standing at room temperature for several days. Crystals did not form even when the solution was concentrated *in vacuo*. Addition of diethyl ether precipitated the remaining original compound as an oil. The liquid was decanted and further addition of ether produced white crystals, which were recrystallized from methanol-diethyl ether. The oil was recycled to produce additional crops of crystals. Yield 0.36 g (78.3%). m.p. = 246° (decomp.). $(\alpha)_D^{25} = +10.2°$ (c = 1.23% in methanol). $C_{28}H_{27}NO_8 \cdot HBr$ (586.45).

Calculated:	57.35% C	4.81% H	2.39% N	21.83% O
Found:	56.78% C	4.69% H	2.39% N	22.31% O

Ethyl 3,4,6-Tri-O-Benzoyl-β-D-Galactosaminide Hydrobromide (V-b). Fifty-hundredths of a gram of (IV) was dissolved in 15 ml of absolute ethanol, four drops of pyridine were added, and the mixture allowed to stand at room temperature. After several days the liquid was filled

with white fluffy crystals. These were cooled, filtered, and washed with diethyl ether. Additional crystals were obtained by concentrating the mother liquor, adding diethyl ether, and separating the crystals from the oily starting material. The combined crops were recrystallized from methanol-diethyl ether. Yield 0.38 g (80.4%). m.p. = 250° (decomp.). $(\alpha)_D^{25} = +14.6°$ (c = 1.16% in methanol).

$C_{29}H_{29}NO_8 \cdot HBr$ (600.47).

Calculated:	58.01% C	5.04% H	2.33% N
Found:	57.82% C	4.97% H	2.32% N

n-Propyl 3,4,6-Tri-O-Benzoyl-β-D-Galactosaminide Hydrobromide (V-c). One and twenty-seven hundredths of a gram (0.002M) of (IV) were added to 200 ml of distilled 1-propanol and the liquid stirred and heated until the solid dissolved. Four drops of pyridine were added and the solution left at room temperature. Concentration by vacuum distillation produced white crystals when nearly dry. The product was recrystallized from methanol-diethyl ether. Yield 0.35 g (28.5%). m.p. = 230° (decomp.).

$(\alpha)_D^{25} = +19.9°$ (c = 1.00% in methanol).

$C_{30}H_{31}NO_8 \cdot HBr$ (614.50).

Calculated:	58.64% C	5.25% H	2.28% N
Found:	58.66% C	5.08% H	2.30% N

Isopropyl 3,4,6-Tri-O-Benzoyl-β-D-Galactosaminide Hydrobromide (V-d). Stirring and slight heating were used to dissolve 1.27 g of (IV) in 200 ml of distilled 2-propanol. Addition of four drops of pyridine, and storage for several days, resulted in the formation of flaky, white crystals. Concentration of the mother liquor produced additional amounts. The product was recrystallized from methanol-diethyl ether. Yield 0.72 g (58.6%). m.p. = 259° (decomp.). $(\alpha)_D^{26} = +23.9°$ (c = 1.18% in methanol). $C_{30}H_{31}NO_8 \cdot HBr$ (614.50).

Calculated:	58.64% C	5.25% H	2.28% N
Found:	58.77% C	5.43% H	2.63% N

n-Butyl 3,4,6-Tri-O-Benzoyl-β-D-Galactosaminide Hydrobromide (V-e). Stirring and slight heating were used to dissolve 1.27 g of (IV) in 200 ml of distilled 1-butanol. Four drops of pyridine were added and the solution left at room temperature. No crystals formed until most of the solvent was removed. Recrystallization from methanol-diethyl ether gave 0.42 g (35.4%) of white flaky crystals. m.p. = 246° (decomp.). $(\alpha)_D^{27} = +21.6°$ (c = 1.06% in methanol). $C_{31}H_{33}NO_8 \cdot HBr$ (628.52).

Calculated:	59.24% C	5.45% H	2.23% N
Found:	58.81% C	5.57% H	2.55% N

Benzyl 3,4,6-Tri-O-Benzoyl-β-D-Galactosaminide Hydrobromide (V-f). Ten milliliters of distilled benzyl alcohol were used to dissolve 0.40 g of (IV), three drops of pyridine were added, and the mixture allowed to stand. No crystals formed. Diisopropyl ether was added to turbidity and the solution allowed to stand until white crystals had formed. These were cooled, filtered, and washed with diisopropyl ether. Additional crystals were obtained by concentrating the solutions back to benzyl alcohol and recycling by heating and stirring the mixture in a water bath at 40-45° for several hours, then crystallizing as before. Recrystallization from methanol-diethyl ether gave 0.16 g (38.4%). m.p. = 247° (decomp.).

$(\alpha)_D^{27} = -21.6°$ (c = 0.30% in methanol).

$C_{34}H_{31}NO_8 \cdot HBr$ (662.55).

Calculated:	61.64% C	4.87% H	2.11% N	19.32% O
Found:	61.19% C	5.08% H	2.51% N	19.59% O

CHAPTER V

SUMMARY

D-Galactosamine has been known as one of the rarer amino sugars, occurring widely distributed in small concentrations in living organisms. The biological importance of D-galactosamine and its derivatives was thought to justify a study of the chemical properties of this group of compounds. As a result of this study the reactions of D-galactosamine have been found to differ more than previously thought from those of D-glucosamine, the more common amino sugar.

For this investigation D-galactosamine hydrochloride was isolated from chondroitin sulfate, a polysaccharide obtained from the cartilaginous tissues of animals. This was changed by appropriate reactions to the 1,3,4,6-tetra-O-benzoyl-N-carbobenzoxy-α-β-D-galactosamine (III). This sirup, when reacted with hydrogen bromide in glacial acetic acid, produced 3,4,6-tri-O-benzoyl-1-bromo-α-D-galactosamine hydrobromide (IV), which was used as the starting material for the subsequent reactions.

The first phase of the project involved synthesizing, by a series of stereospecific reactions, both the alpha and beta anomers of the tetra-O-benzoyl-N-benzoyl-and the tetra-O-benzoyl-N-carbobenzoxy-D-galactosamine series of

derivatives. The properties of these pure anomers were studied with a view toward developing a method of separating the respective alpha and beta anomers from each other in a mixture.

The second phase involved the preparation, by a non-stereospecific reaction, of an anomeric mixture of each of the two series mentioned above. Then, using the information acquired by a study of the pure forms, each of the two sirups was separated into its pure alpha and beta components. In so doing, a thin-layer chromatographic method was developed to determine the anomeric purity of the product obtained. In addition, the results were transferred to column chromatography, and this method was used to remove the final traces of impurities. The melting point and specific optical rotation were determined for each compound. Moreover, each of the anomeric pairs was compared to each other by mixed melting point, thin-layer chromatography, and infrared spectra.

The third and final phase of the investigation involved the reaction of the 1-bromo compound (IV) with six different alcohols to obtain a series of beta glycosides. The physical properties of this group of compounds are reported.

Certain difficulties in the synthesis and stability of the various reported compounds are discussed. Also, a

limited rate investigation of the benzoylation of N-carbobenzoxy-D-galactosamine is reported to explain the relative amounts of the two anomers obtained from the separation in this series. These observations support the concept that anomeric equilibration reactions may well be of more common occurrence than has formerly been believed.

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