DERIVATIVES OF
CHONDROITINSULFURIC ACID

DISSertation
Presented in Partial Fulfillment of the Requirements
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By

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Approved by:

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DERIVATIVES OF
CHONDROITINSULFURIC ACID

I. INTRODUCTION AND STATEMENT OF PROBLEM

Heparin, a mucopolysaccharide, possesses the unique physiological property of preventing the clotting of blood in the human system. Much research has been conducted regarding the chemical composition of this polysaccharide as well as the structural features of the material which are responsible for this physiological action. Evidence has been presented which indicates that the anticoagulant activity of heparin is concerned with the presence of O-sulfate groups throughout the polymer, the molecular weight of the material, the structure of the carbohydrate repeating unit present in the polymer and the presence of sulfamate groups throughout the polymer chain.

The purpose of this investigation is many fold. It is concerned with the preparation of a synthetic anticoagulant possessing the properties of heparin and of a structure similar to that of heparin. This objective necessitated a study of the fundamental chemistry of chondroitinsulfuric acid, a mucopolysaccharide. The synthesis of a material similar to heparin required the preparation of pure chondroitinsulfuric acid, of
N-deacetylated chondroitinsulfuric acid, and of N-deacetylated and sulfated chondroitinsulfuric acid. Another purpose of this investigation is the elucidation of some of the details of the structure of chondroitinsulfuric acid which are not understood at this time. This objective required the synthesis of certain chemical derivatives of this polysaccharide and their chemical evaluation.

The final purpose of this investigation is the study of the fundamental chemistry of the sulfamate group by means of suitable model compounds containing this group. It was considered desirable to conduct a study of the infrared spectral characteristics of this general class of sulfamate compounds so as to relate further their chemical similarities.
II. HISTORICAL

A. Heparin

Heparin was discovered by Jay McLean while working in William H. Howell's laboratory in 1916 (1). McLean was engaged in the purification of the phosphatide cephalin. This material, which exerted a strong thrombo-plastic (coagulant) action on blood, was being prepared from the brain and other organs and was being subsequently extensively purified. At this time two other "phosphatides" were prepared by McLean by separation from cephalin by means of their insolubility in boiling alcohol. These were the heart phosphatide courin and the liver phosphatide previously described by Erlandsen and Baskoff, respectively. However, McLean found these materials prevented or prolonged the coagulation of dog plasma, which was contrary to expectations. The study of this material was continued and at the end of the first year Howell gave a preliminary report on the findings. On April 7, 1917, in a Harvey Lecture, Howell reported that the substance, if present in sufficient concentration, would prevent the in vivo and in vitro coagulation of blood. He reported that one milligram
of the substance would prevent the coagulation of 1 to 2 ml. of blood for 24 hours when stored at 0°C. He also stated that the blood of dogs, injected intravenously with the material to the extent of 100 mg. of the material per kilogram of body weight, would not clot. At this time the material had been isolated from liver, heart, muscle, lymph nodes and uterine mucous membrane.

In 1918 Howell and Holt (2) presented a more detailed characterization of the material and named it heparin because of its abundance in beef liver. In 1924 Howell (3) showed that the material did not contain phosphorus but did contain sugar. This material, prepared by a new procedure, gave a positive Molisch test but was reported to be free of sulfur.

In 1928 Howell (4) presented an improved preparative procedure in which aluminum silicate was used for the removal of inactive material and the active material was
further purified by precipitation with excess barium hydroxide solution. At this time more details on the chemistry and properties of heparin were presented. Howell stated that the material contained uronic acid and that hydrolysis of the material with hydrochloric or nitric acid brought about the crystallization of calcium sulfate.

In 1933, Charles and Scott (5) presented a preparative method in which the yield of heparin from tissue was greatly improved. These workers extracted ground and autolyzed beef liver with alkaline ammonium sulfate and coagulated the mass by heating the mixture to 70°C. The heparin was precipitated from the filtered extract by adjustment to the solution to pH 2. These workers also noted that heparin was present in considerable quantities in lungs and was present throughout the body. The purest preparations of heparin by these workers contained 25-33% ash and about 2% nitrogen. However, they did not get a positive naphthoresorcinal test for uronic acid, as did Howell, and therefore left the question of the chemical composition of heparin open. In the same year Fischer and Schmitz (6) reported the preparation of a very pure

(5) A. F. Charles and D. A. Scott, J. Biol. Chem., 102, 431 (1933).
heparin through the brucine salt. They reported heparin to be a nitrogen-free polysaccharide with the composition $C_{18}H_{32}O_{17} \cdot 6H_2O$.

The next significant advance in the chemistry of heparin was made by Jorpes (7). At this time this worker

reported the analysis of heparin preparations from beef and horse liver according to the Tollens-Lefèvre procedure. The material was hydrolyzed with hydrochloric acid and the carbon dioxide evolved was collected in a Pregl-tube containing barium hydroxide. This was a quantitative procedure. The following year Jorpes (8)

published his findings with regard to the hexosamine content of heparin. This worker utilized the hexosamine color reaction developed by Zuckerkandle and Meissner-Kleberman (9) and made quantitative by Elson and Morgan (10)
This reaction was based on the intensity of the color produced by the reaction of p-dimethylaminobenzaldehyde on hydrochloric acid and alkali-treated N-acetylglucosamine. Using this quantitative procedure Jorpes was able to show that heparin contained one molecule of uronic acid for each molecule of amino sugar. These two components accounted for 40% of the composition of purified heparin if the amino sugar were assumed to contain an N-acetyl group. The ash content of this material was found, at this time, to be about 40-45% of the total. A study of the ash showed it to contain magnesium and calcium sulfate. At this point heparin had a composition closely resembling chondroitinsulfuric acid but had an apparently higher sulfate content. Free sulfates were removed by barium chloride precipitation and esterbound sulfate was freed from the heparin by acid hydrolysis followed by barium chloride precipitation. Esterbound sulfate was found to be equivalent to 7-8% sulfur in the material.

In two papers Charles and Scott (11,12) published
a detailed procedure for the preparation of pure barium acid heparinate from minced beef lung. Their procedure made use of alkaline ammonium sulfate extraction, removal of impurities with Lloyd's reagent, trypsin digestion for removal of protein, cadmium chloride treatment for removal of impurities and purification through the benzidine salt. These workers stated that their final purified product was crystalline. They published the following analysis for this barium acid heparinate: C, 18.6%; H, 3.9; N, 1.68; S, 9.3; ash, 33.6; Ba, 19.8; amino-N (Van Slyke), 0.4. They considered that glucuronic acid was absent in the material and showed that its anticoagulant activity was destroyed by formaldehyde or nitrous acid. Meyer and Smyth (13) analyzed this material and obtained the following results: N, 2.36; amino sugar, 16.3; uronic acid, 20.1; acetyl, 2.2; sulfur, 9.8; ash, 33.3, equivalent weight of the acid salt, 749. These workers considered it to be a polysulfuric acid ester of mucoitinsulfate.

During the period from 1938-1943 various workers...
published analytical results on their preparations of heparin. Complications arose due to the fact that fractional precipitation of heparin benzidine salts yielded heparin fractions of varying sulfur content and there was disagreement concerning the apparent acetyl content of the material.

In 1937, Jorpes and Bergström (14) stated that purified sodium heparinate, on hydrolysis at 100°C with aqueous sulfuric acid yielded considerable quantities of acetyl. Reinert and Winterstein (15) analyzed neutral sodium heparinate, prepared from crystalline barium acid heparinate, and found it to contain 10% acetyl.

Charles and Todd (16), in a further study of their crystalline barium acid heparinate, stated that the material was hydrated and contained 12.1% water. They reported the following analysis on the hydrated basis: C, 17.8; H, 3.1; N, 1.9; S, 9.6; Ba, 19.7;
ash, 33.4. Recalculated on the anhydrous basis the values were: C, 20.2; H, 3.5; N, 2.2; S, 11.0; Ba, 22.5. Their values for the acetyl content varied from 1.5 to 3.0%.

Masamune, Suzuki and Kondoh (17) reported the following analysis for a barium acid heparinate prepared from beef lung: hexosamine, 26.4; hexuronic acid, 28.7; N, 2.1; acid-hydrolyzable sulfur, 8.7; acetyl, 0.0; amino-N, 0.0; ash, 27.7.

However, in 1937, Jorpes and Bergström (14) stated that purified heparin, on hydrolysis at 100°C. with aqueous sulfuric acid, yielded considerable quantities of acetyl.

Wolf from and coworkers (18,19), conducted a rather intensive analytical investigation of the crystalline barium acid heparinate of Charles and Scott. These workers found no acetyl groups in this material. They hydrolyzed barium acid heparinate at 100°C. in 20% p-toluenesulfonic
acid in a modification of the Freudenberg and Harder (20)

(20) K. Freudenberg and M. Harder, Ann., 433, 230 (1923).

procedure. Likewise, they found none on application of
the Kuhn-Roth (21) chromic acid oxidation for the detec-

(21) R. Kuhn and H. Roth, Ber., 66, 1274 (1933).

tion of the CH$_3$-C grouping. Simultaneously with the
heparin characterization, these workers analyzed purified
sodium chondroitinsulfate and sodium acid mucopolysaccharide.
In these materials, on application of the above acetyl
analysis procedures, acetyl was quantitatively determined
in both cases. A complete summary of their findings was
presented in their first cited publication. Neutralization
equivalents (electrometric) were determined for
barium acid heparinate, sodium and mucoitinsulfate and
barium acid chondroitinsulfate. These were found to
be 800±50, 500 and 490, respectively. Optical rotations
were determined on the above materials and on crystalline
barium acid heparinate (prepared through the benzidine
salt). The $\overline{[\alpha]}_{\text{D}}^25+25$ values for these were
found to be: +44°, -7.4°, -11.5° and +47.5°, respectively.
A brief summary of their findings is presented in Table I.
<table>
<thead>
<tr>
<th>Constituent</th>
<th>Barium Acid heparinate %</th>
<th>Molar Ratio</th>
<th>Sodium heparinate(a) %</th>
<th>Molar Ratio</th>
<th>Sodium mucoitin-sulfate %</th>
<th>Molar Ratio</th>
<th>Sodium chondroitin-sulfate %</th>
<th>Molar Ratio</th>
</tr>
</thead>
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<tr>
<td>Anhydrohexosamine</td>
<td>17.7±1.3</td>
<td>1.0</td>
<td>21.4±1.5</td>
<td>1.0</td>
<td>29.2±2</td>
<td>1.0</td>
<td>31±2</td>
<td>1.1</td>
</tr>
<tr>
<td>Anhydrohexuronic-acid</td>
<td>17.9</td>
<td>0.9</td>
<td>20.5</td>
<td>1.0</td>
<td>29.0</td>
<td>1.1</td>
<td>31.6</td>
<td>1.0</td>
</tr>
<tr>
<td>N-acetyl (as CH₂CO)</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ester sulfate (as SO₃)</td>
<td>28.2</td>
<td>3.0</td>
<td>32.3</td>
<td>3.0</td>
<td>14.7</td>
<td>1.0</td>
<td>14.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium Barium</td>
<td>23.6</td>
<td>1.5</td>
<td></td>
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88.4%  86.6%  89.8%  95.5%

(a) Calculated to this basis from data obtained on the crystalline barium acid salt.
These workers stated that the amino group of heparin was not acetylated and was not free (Van Slyke and ninhydrin test). They found that purified sodium heparinate consumed slightly more than one milliequivalent of periodate per gram of material. This quantitative determination indicated an equivalent weight of 1900 for sodium heparinate which in turn indicated a tetrasaccharide repeating unit for the material.

These workers identified D-glucosamine as an acid hydrolysis product of barium acid heparinate. They presented rate studies of the behavior of crystalline barium acid heparinate, sodium chondroitinsulfate and sodium mucosulfate in the Van Slyke determination.

A complete crystallographic study of crystalline barium acid heparinate definitely showed it to be crystalline. Further, these workers recrystallized barium acid heparinate (not prepared through the benzidine salt). These workers recrystallized the material five times from hot acetic acid. In this work they presented very definite data which showed that the anticoagulant activity of the material was progressively destroyed in each recrystallization until the fifth recrystallization product was essentially inactive. Further, they noted that in this process the amino group became free. At the end of five recrystallizations the inactive material contained 1.1% free amino nitrogen (Van Slyke), although the other
analytical data did not change significantly (C, H, N, ash, toluidine blue test). They also found that prolonged drying of the material deactivated it.

Wolfrom and Karabinos (22) conducted a series of hydrolysis studies of barium acid heparinate. These workers employed 4N hydrochloric acid at 98°C. and heparin concentrations of 1.5% or less for the hydrolysis. These conditions had been shown, in the previous publication (18), to be sufficient to completely liberate the sulfate and hexosamine without destroying the latter. These hydrolysis rate studies were presented and the percent of reducing sugar liberated, change in optical rotation, percent sulfur lost, anticoagulant activity decrease, and percent hexuronic acid destroyed in the hydrolysis of heparin was shown. These workers showed that the uronic acid was destroyed almost as fast as it was liberated, that the sulfate was hydrolyzed rapidly, that the anticoagulant activity of the material was completely lost within fifteen minutes, that the percent reducing sugar present reached a constant value after ten hours and that the maximum in the optical rotation corresponded roughly to the maximum in the reducing sugar curve (glucosamine). The previously determined
rate of hexosamine liberation was included in the data and was shown to parallel roughly the rate of uronic acid destruction. No new constituents of barium acid heparinate were found during the study.

One of the most significant findings with respect to the chemistry of heparin was published by Wolf from and McNeely (23). These workers were able to relate the anticoagulant activity of heparin to the amino group present in the material. In a prior publication (18), it had been shown that recrystallization of barium acid heparinate from hot acetic acid brought about a decrease in the anticoagulant activity of the material. This (23) publication was a quantitative study of that phenomenon. A 2% solution of barium acid heparinate in 11% acetic acid was maintained at 68±2°C. for several hours. At intervals, samples were withdrawn and tested for anticoagulant potency. Also, crystalline barium acid heparinate was recovered from the above solution at intervals and tested for anticoagulant potency. The values of the latter were in line with those of the former procedure. The anticoagulant activity was found to decrease with time and after 48 hours the material was
biologically inactive. Control experiments with sodium heparinate in 11% acetic acid gave comparable results and showed that barium did not play a part in the deactivation. That acetic acid was not necessary for the deactivation was shown by the fact that a solution of the barium acid salt (pH 3.6 at 25°C.) became deactivated on standing at 68°C. due to its own acidity. Further, it was found that sodium heparinate, buffered to a pH of 2.6 at 25°C., became inactivated in a parallel manner, showing that deactivation was solely a function of pH.

The inactivation process was followed by reducing value determinations, optical rotations and relative viscosity determinations. No significant changes in these properties were noted.

The inactivation process was followed by Van Slyke analysis for amino nitrogen. An inverse relationship between activity and initial (nitrogen evolution in the first five minutes) Van Slyke amino nitrogen was found. An increase in amino nitrogen with time was almost exactly parallel to the rate of loss of activity of the material. Therefore, it was shown that the nitrogen bond was very significant in the activity of the barium acid heparinate. The anticoagulant activities were determined by the Foster (24) modification of the procedure.

of Reinert and Winterstein (15).

In 1946, Wolfrom and Rice (25) subjected barium acid heparinate to oxidative hydrolysis. This was accomplished by treating a cooled aqueous solution of the material with bromine and sulfuric acid. The solution was allowed to stand at 3°C. for one week and from the reaction mixture these workers isolated D-glucaric acid (as the crystalline potassium acid salt) and crystalline D-glucosaminic acid, both in fair yield. These results established D-glucuronic acid as the uronic acid component of heparin.

When submitted to electrophoretic resolution, the biologically active heparin was found to move as one mobile phase (26). Purified sodium heparinate, prepared through the benzidine and the crystalline barium acid salts, was desulfated and acetylated by means of acetic anhydride in cold absolute sulfuric acid (27). The


product of this reaction was soluble in water and in several organic solvents. It was reducing to Fehling solution and did not contain sulfur. It was characterized as the amorphous sodium acid salt of \( \text{CHCl}_3 \). Treatment of this material with barium methoxide gave N-acetyl desulfated heparin whose amorphous barium salt, \( \text{CHCl}_3 \) (water) consumed 1.0 mole of periodate per disaccharide unit without the formation of formaldehyde or formic acid (28). Partial (28) M. L. Wolfrom, R. Montgomery, J. V. Karabinos and P. Rathgeb, J. Am. Chem. Soc., 72, 5796 (1950).

acid hydrolysis of this material gave an amorphous reducing disaccharide, \( \text{CHCl}_3 \) (water), isolated through its amorphous cupric salt. This compound, designated heparosin sulfurous acid, contained one sulfate ester group, hexosamine and hexuronic acid with 0.1 of the latter free. With controlled periodate assay the compound consumed 3.0 moles of periodate per mole of compound with the formation of one mole of formic acid and no formaldehyde. Both the hexosamine and the uronic acid portions were destroyed in the reaction. Heparosin sulfurous acid was acetylated with acetic anhydride and silver acetate to yield the N-acetyl derivative. With controlled periodate assay one mole of this material consumed two moles of periodate and one mole of formic
acid and no formaldehyde formed. Only the hexuronic acid portion of the molecule was destroyed in the reaction.

On the basis of this work, the authors proposed a highly probable formula (I) for heparosinsulfuric acid. On the basis of this and the work of Jorpes and associates (29), these workers also suggested a formula for the tetrasaccharide repeating unit of barium acid heparinate (II). On the basis of the strongly positive optical rotation of heparin and its derivatives, these workers considered that the α-D linkage was probable.

\[ \text{I} \]
They considered the O-sulfate placement in the D-glucuronic acid component limited to one of the four secondary hydroxyl groups in the tetrasaccharide repeating unit. They stated that alkali-treated heparin showed, by periodate consumption, 2 α-glycol groups per tetrasaccharide unit.

Although Wolfrom and McNeely (23) (see also Masamune and coworkers (17)) suggested the possible presence of a sulfamic acid linkage in heparin, Jorpes, Boström and Mutt (29) were the first to adduce good evidence for it. These workers compared the acetyl content of heparin, sodium chondroitinsulfate and chitin by means of the Clark (30) distillation procedure. By this method they

\[ \text{(30) E. P. Clark, "Semimicro Quantitative Organic Analysis", New York, N. Y., Academic Press, Inc. (1943).} \]

found less than 1 per cent acetyl present in heparin. Analysis by means of chromic acid oxidation (31) showed
that the material contained less than 1 per cent acetyl and that better purified samples contained still less. From these findings these workers concluded that the amino group of heparin was not substituted by acetyl. These workers cited the work of Wilander (32) who showed

that the free acid of heparin, purified by electrodialysis, consumed four equivalents of alkali per period for neutralization. Previous work had shown that hydrolysis of heparin with 7.5 per cent (by volume) sulfuric acid for 10 minutes destroyed the anticoagulant activity of the material but reducing groups were produced only to the extent of 1-2 per cent of the organic material present (13). This evidence excluded the possibility of the amino group in heparin being joined to carbonyl functions since the amino group was freed on acid hydrolysis.

These workers studied the rate of release of free amino and sulfate groups when heparin was hydrolyzed with dilute hydrochloric acid. They found no strict relationship and noted that difficulties were encountered
due to the polymeric heparin present in the solution causing the barium sulfate precipitate to be colloidal in nature. On treatment with 0.04 N hydrochloric acid at 100°C., they found that all of the anticoagulant activity of heparin was destroyed in 1 hour and 75 per cent was destroyed in 5 minutes. No free amino, or very little, was produced on boiling heparin with N sodium hydroxide for 1 hour. From the alkaline hydrolysis these workers isolated a sodium salt of heparin containing 9.5 per cent sulfur and 0.31 per cent amino nitrogen. This material was neutralized and dialyzed for purification and submitted to hydrolysis with 0.04 N hydrochloric acid. Rate studies of this acid hydrolysis showed a close relationship between sulfate release and the appearance of free amino groups in heparin. These workers noted the similarity of the reactions of heparin on treatment with acid and base as compared to sulfamic acid. As a result of their studies these workers concluded that heparin contained a sulfamic acid group.

Meyer and Schwartz (33) in their studies of the structure of heparin, concluded that this polysaccharide contained N-sulfate and O-sulfate groups. These workers found only 10 per cent of the amino groups to be free
in Roche commercial heparin (protein-free) when this material was submitted to the Van Slyke (34) analysis.


Acetyl analysis of this material by means of acid hydrolysis followed by distillation of the volatile acids showed that heparin did not contain acetyl or formyl groups. A very careful analysis showed that about 0.25 acetyl groups per disaccharide unit were present in "Roche" heparin before electrophoresis. On submitting the material to this purification it was found that the acetyl content was substituted in an immobile component which was present in the starting material. Treatment of heparin with nitrous acid did not lower the viscosity of the polymer solution. On acid hydrolysis with N hydrochloric acid, these workers found that the amino group of heparin was freed at roughly the same rate as D-glucosamine N-sulfate as compared to D-glucose 6-sulfate hydrolysis. These workers stated further that heparin formed a nitroso compound with nitrous acid analogous to sulfamates. This was partially substantiated by analysis of the product of the reaction. These contained more nitrogen than the starting heparin but slowly lost it on standing. This work further confirmed the concept of a sulfamic acid linkage in heparin.
The findings of the three groups of workers cited (28, 29, 33) constitute, in the main, the present status of the chemical structure of heparin. Publications concerning the physiological aspects of heparin have been released since that time. Jorpes and coworkers (35)


have confirmed further their earlier hydrolysis studies of heparin. Stacey and coworkers (36) have presented


evidence, based upon the colorimetric measurement of free amino after its interaction with fluoro-2,4-dinitrobenzene, which indicated that there was no direct relationship, on acid hydrolysis, between sulfate release, appearance of free amino and anticoagulant activity loss in heparin. These workers suggested other structural possibilities (cross linkages) to account for the anticoagulant activity of heparin.

A rather interesting finding has been noted recently with regard to heparin. Marbet and Winterstein (37) have

isolated a new polysaccharide accompanying heparin in bovine lung. These workers reported that two chondroitinsulfuric acids accompanied heparin. One was the well-known chondroitinsulfuric acid of $\Delta_\alpha D -30^\circ$. The other was a chondroitinsulfuric acid, isolated as the zinc salt, which had an $\Delta_\alpha D -60^\circ$ and an anticoagulant activity of 36 I.U./mg. This material was stated to contain sulfuric acid, acetic acid, uronic acid, galactosamine. These workers reported that this material had the empirical formula $(C_{14}H_{19}O_{14}NSNa)_x$, the same as chondroitinsulfuric acid sodium acid salt. This new material they designated as B heparin. These workers also isolated a similar material from sheep lungs with an $\Delta_\alpha D -67^\circ$ and an activity of 70 I.U./mg.
B. Chondroitinsulfuric Acid

Chondroitinsulfuric acid is a mucopolysaccharide occurring in various cartilages (38-42), skin (43), umbilical cord (44), scar tissue (45), connective tissue (46) and cornea (47). This polysaccharide has been extensively studied by numerous workers since Müller (48) began his studies of animal glues. This polysaccharide was first prepared in reasonably pure form by Krukenberg (49).

(38) E. Winter, Biochem. Z., 246, 10 (1932).
(40) T. Miyazaki, J. Biochem. (Japan), 20, 211, 223 (1934).
(43) K. Meyer and Elizabeth Chaffee, J. Biol. Chem., 128, 491 (1941).


(48) J. Müller, Ann., 21, 277 (1837).
Numerous procedures have been published for the preparation of this material free of protein collagen and undegraded from its original state.

An excellent review of the chemistry of chondroitin-sulfuric acid was prepared by K. K. Madison (50) and need not be repeated here. However, a brief statement of the pertinent facts concerning its structure at that time should be given. The component monosaccharide units of chondroitinsulfuric acid were known to be D-glucuronic acid (18,51,52), N-acetylchondrosamine (53,54).

The material was known to contain one sulfate group per disaccharide unit. The disaccharide repeating unit of chondroitinsulfuric acid was first isolated as its crystalline ethyl ester hydrochloride by Hetting (55).
Levene (56) prepared the crystalline methyl ester of this disaccharide (chondrosine) as the hydrochloride and converted this to what he considered to be methyl 2-amino-2-deoxy-D-galactosyl-L-gulonate. In a series of reactions this worker prepared the crystalline methyl octaacetyl-2-amino-2-deoxy-D-galactosyl-L-gulonate, crystalline methyl heptamethyl-N-acetyl-2-amino-2-deoxy-D-galactosyl-L-gulonate and heptamethyl-N-acetyl-2-amino-2-deoxy-D-galactosyl-D-glucitol. This worker also prepared methyl hexamethyl-N-acetyl-2-amino-2-deoxy-D-galactosyl-L-gulonate. However, the death of Dr. Levene interrupted this work and the structure of chondrosine established by him.

Bray, Gregory and Stacey (52) prepared a degraded methylated chondroitin from chondroitinsulfate. Hydrolysis of this material led to the isolation of methyl 2,3,4-tri-O-methyl-α-D-glucuronoside, methyl N-acetyl-3,4,6-trimethylchondrosaminide methyl N-acetyl-di-O-methyl-α-D-chondrosaminide and methyl di-O-methyl-d-glucuronoside. From the relative ratios of the above
fragments these workers suggested a trisaccharide repeating unit for chondroitinsulfate which was branched. These results were not considered definite in that a degraded chondroitinsulfate was employed and these workers disregarded the fact that the ratio of glucuronic acid to galactosamine was 1 to 1 in chondroitinsulfuric acid.

Meyer, Odier and Siegrist made some rather important contributions to the chemistry of chondroitinsulfate (57).


These workers found chondroitinsulfate to have a molecular weight of about 30,000 when prepared by mild alkaline extraction, much in agreement with the findings of Blix and Snellman (58). Both groups of workers found chondroitinsulfate to have a non-branched structure as compared to the findings of Stacey and coworkers (52). Meyer and coworkers found chondroitinsulfate to be composed of equal numbers of chondrosamine and D-glucuronic acid residues as well as containing one sulfate group per disaccharide unit. These workers conducted periodate
studies on the purified chondroitinsulfate, partially desulfated and degraded chondroitinsulfate, the product obtained from methanolytic scission of completely methylated chondroitinsulfate and the product obtained from the acid hydrolysis (partial) of methylated chondroitinsulfate. From the results of these and other experiments, these workers stated that the chondrosamine and D-glucuronic acid residues of chondroitinsulfate were joined in β-D-(1→3) glycosidic linkages. The proposed repeating unit was as follows:

It should be stated that these workers did not adequately describe the intermediates in their periodate studies. The assignment of this structure to the repeating unit was supported later by the findings of Masamune and coworkers (59) on the basis of a positive Elson-Morgan

reaction and periodate oxidation of N-acetyl-c-ethyl ester.

Wolf from and coworkers (60) published the

(60) M. L. Wolf from, R. K. Madison and M.

their investigation of the structure of chondrosine, furic acid. These workers approached the problem from the standpoint of structural studies of the di-
repeating unit, chondrosine, of the same genus as that begun by Levene (56). They prepared pure sodium chondroitinsulfate from crude commercial chondroitinsulfuric acid which in turn was obtained from cartilage (x).


Purification was accomplished by filtration of an aqueous solution of this material through Filter followed by precipitation from water with glacial acetic acid and 95 per cent ethyl alcohol, respectively. The so-isolated sodium acid salt was converted to the salt by neutralization with alkali. The sodium salt was purified further by precipitation from water means of the addition of several volumes of 95 per cent ethyl alcohol followed by adequate washing with
cent ethyl alcohol, absolute ethanol and ethyl ether. The product was dried in vacuo over P₂O₅ and was found to give a negative biuret test for protein and was free of inorganic sulfate. The sodium salt was converted to the barium salt by passage through Amberlite IR-100 (x)


cation exchange resin (acid form) to yield an aqueous solution of the free acid. Admixture of this solution with one containing the calculated amount of barium acetate led to the isolation of the barium salt of chondroitin-sulfuric acid. Electrophoretic studies of this material showed it to be pure. Drying of the material in vacuo over phosphoric anhydride yielded a white amorphous powder \( \alpha_{\text{7,0}} -24^\circ \) (c 2.1, water).

Chondrosine hydrochloride was prepared from barium chondroitin sulfate by partial hydrolysis of the latter with acid (56). Chondrosine hydrochloride was converted to the crystalline methyl ester hydrochloride (V) according to the procedure of Levene (56). After one recrystallization from absolute ethanol this material had a m.p. 155-156°, \( \alpha_{\text{7,5}} +39^\circ \) (c 4, methanol, no mutarotation, 5 hours). Analysis of this material gave: C, 38.06; H, 5.90; N, 3.49; OCH₃, 8.27; equiv. wt., 418; in rather close agreement for C₁₂H₂₁O₁₀NCl (OCH₃).
This compound was converted to what was considered to be methyl hepta-0-acetyl-4-0-(N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-L-gulonate (VI) by reduction with raney nickel followed by esterification of the carboxyl group and acetylation. The product of this series of reactions had a m.p. of 121-123°; $\alpha_{D}^{28} = -23°$ (c 1.8, ethanol) and exhibited the following analysis: C, 49.22; H, 5.70; N, 2.10; OCH$_3$, 4.85; CH$_3$CO, 48.6. The analytical values were in good agreement with the formula C$_{14}$H$_{17}$O$_{11}$N(CH$_3$CO)$_7$(OCH$_3$) as established by Levene (56).

Crystalline chondrosine hydrochloride methyl ester was benzoylated to yield hexa-0-benzoyl-N-benzoylchondrosamine methyl ester, m.p. 126-129°, $\alpha_{D}^{22} = +83°$ (c 2, chloroform). The yield was 26 per cent of theory. These workers prepared 4-0-(N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-L-gulonamide (VII) from methyl 4-0-(hepta-0-acetyl-N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-L-gulonate (VI) by deacetylation of the latter compound in methanolic ammonia. An amount of 3.45 g. of starting material yielded 1.82 g. of product. The amorphous product was crystallized from methanol-chloroform; yield 1.50 g., m.p. 132-134°, $\alpha_{D}^{27} = -100°$ (c 1.9, water). Analysis of the material showed: C, 42.21; H, 6.91; N, 7.09; CH$_3$CO, 11.0; in good agreement with the empirical formula C$_{12}$H$_{23}$O$_{10}$N$_2$(CH$_3$CO). An amount of 0.9937 g. of this compound of oxidized at 12°C. in 100 ml. of
solution containing 5.28 millimoles of sodium meta-periodate. All of the periodate had been reduced after fifteen hours and from this solution an amount of 0.313 g. of crystalline 2-O-(N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-D-erythruronamide (VIII) was obtained (dried in vacuo at 78°) by passage of the solution through Amberlite exchange resins IR-4 and IR-100, respectively. The material had a m.p. of 177-179°, \( \alpha_{D}^{27} -77° \) (c 1.5, water). Analysis of this material showed it to contain: C, 42.87; H, 5.29, N, 8.08; CH\(_3\)CO, 13.1; in good agreement with the formula C\(_{10}\)H\(_{17}\)O\(_6\)N\(_2\)(CH\(_3\)CO).

These workers thus found that the diamide alditol, 4-O-(N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-L-gulonamide, consumed two moles of periodate, in the reduced portion, to yield one mole of formaldehyde, one mole of formic acid, and 2-O-(N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-D-erythruronamide with the N-acetamido-2-deoxy-D-galactopyranosyl radical intact. On longer standing in periodate solution, it was found that one additional mole of periodate was consumed by the latter compound, presumably in cleaving the glycol grouping in this compound. On the basis of these findings, these workers assigned chondrosine the structure designated as 4-O-(2-amino-2-deoxy-\( \beta \)(D)-galactopyranosyl)-D-glucuronic acid. The proposed formulas for the above sequence of reactions are shown here:
These workers found the purified sodium chondroitin-sulfate (S, 6.01; α_728D -24°) consumed one mole of periodate per disaccharide unit at 26° and initial pH of 5.5 in solution. From this solution a material was isolated which exhibited a negative Dische hexuronic acid test (61) and a positive hexosamine color test (62).


This material gave a positive test for acid-hydrolyzable sulfate. These results would tend to indicate that chondroitinsulfate was a linear polymer (due to the stated high yield of 26.7-29.3 g. of chondrosine hydrochloride from 50 g. of barium chondroitinsulfate consisting mainly of chondrosine repeating units. These results also indicated that the linkage between the D-glucuronic acid and the chondrosamine in this repeating unit was 1→4 as shown. Negative optical rotation of the chondrosine derivatives indicated a β-D disaccharide linkage. Further, the results of the periodate oxidation indicated that the uronic acid component of chondroitinsulfate contained the glycol grouping.

A recent publication of Davidson and Meyer (63) is

not in agreement with the conclusions reached by Wolf from and coworkers with regard to the structure of chondrosine and chondroitinsulfuric acid. The findings of Davidson and Meyer had some support in the findings of Masamune (59).

Davidson and Meyer prepared calcium chondroitinsulfate from tracheal cartilage by the procedure of Meyer and Smyth (64). This material exhibited the following analysis: CO₂, 30.7; hexosamine, 27.0; H, 2.61; \( \Delta_2^2D -28^\circ \) (c 2, water). The calcium salt was converted to the barium salt by passage of the aqueous solution through a Dowex-50 (H⁺ form) ion exchange column into the theoretical amount of barium acetate followed by lyophilization. Chondrosine (X) was prepared by acid hydrolysis of barium chondroitinsulfate followed by purification by fractionation on a Dowex-50 (H⁺ form) column. The yield was 67 per cent (based on uronic acid). The material analyzed as follows: H, 3.98; amino N (ninhydrin), 3.92; uronic acid, 54; hexosamine, 42; eq. weight, 352.0; \( \Delta_2^2D +42^\circ \) (amorphous, c 2, water); \( \Delta_2^2D +40^\circ \) (crystallized, c 1, 0.05 NHCl). The above data was in agreement with the formula \( C_{12}H_{21}O_{11}N \).

Chondrosine methyl ester hydrochloride (XI) was
prepared by treatment of chondrosine with cold methanolic hydrochloric acid (65). The yield was 81 per cent of


theory. The melting point and optical rotation of this crystalline material were in agreement with those found by Levene (56) and by Wolfrom (60). 3(?)-0-[(3-D-Gluco-pyranosyl)-2-deoxy-2-amino-D-galactitol (XIII) was prepared from chondrosine methyl ester hydrochloride by reduction of the latter with sodium borohydride in borate buffer. Purification was accomplished by adsorption of the material on a Dowex-50 (H+ form) column elution with acid and neutralization with barium hydroxide. The final product, obtained in excellent yield, contained less than 1 per cent uronic acid, 49 per cent glucose and gave a negative Elson-Morgan (10) and a negative Harding McLean (66) ninhydrin reaction. Glucose was the


only reducing sugar isolated from the hydrolyzate on hydrolysis of this material with N sulfuric acid at 100°C. for seven hours.

The N-acetyl derivative (XV) of the above compound was prepared by utilizing the preferential acetylation
procedure of Roseman and Ludowieg (67). This product,


obtained in 260 mg. yield from 300 mg. of starting material, was found to contain 45 per cent glucose (anthrone) but gave a negative reducing sugar test and a negative test for the free amino group. These facts indicated that the carboxyl function in the glucose portion of this disaccharide was blocked in the glycosidic linkage. Furthermore, \( \beta \)-glucosidase (emulsion) hydrolyzed the above compound in 0.1M, pH 5.0, acetate buffer with the liberation of 15 per cent of the theoretical amount of reducing sugar in 48 hours. On analysis of the hydrolysate by paper chromatography only glucose was found, \( \alpha \)-Glucosidase did not hydrolyze the compound. This N-acetyl derivative was hydrolyzed for 4 hours at 100°C. with 5 ml. of \( N \) sulfuric acid. The amino sugar portion of the compound was removed by passage of the hydrolysate through a Dowex-50 (\( H^+ \) form) column. On evaporation of the eluent and washing to dryness followed by drying in vacuo, a glossy material was obtained. This was acetylated with sodium acetate and acetic anhydride at 100°C to yield 110 mg. (59 per cent yield) of crystalline \( \beta \)-D-glucose pentaacetate, \( \left[ \alpha \right]_D^{26} +2^\circ \) (c 1, CHCl\(_3\)). Admixture of this crystalline material with an authentic
sample of β-D-glucopyranose pentaacetate caused no depression of the melting point of 133.5-134°C.

An amount of 340 mg. of chondrosine was reduced with sodium borohydride in aqueous sodium carbonate solution followed by adsorption of the product on a Dowex-50 (H⁺ form) column. The desired product was displaced from the column with pyridine and the uronic acid containing eluents combined and lyophilized to yield 310 mg. of product which contained less than 1 percent reducing sugar, had a uronic acid content of 52 percent (carbazole) and gave a negative Elson-Morgan test for free 2-amino-2-deoxy sugar.

An amount of 300 mg. of this reduced disaccharide (VII) was esterified with cold methanolic hydrochloric acid and acetylated with cold acetic anhydride in pyridine to yield, on crystallization from ethanol, 121 mg. of product. On recrystallization from ethanol this product had m.p. 120.5-121°C (cor.); $\Delta T^{23}_D -21°$ (c 1, CHCl₃). For this compound, which these workers considered to be methyl hepta-0-acetyl-3(?)-0-)β-D-glucopyranosyl uronate)-2-acetamido-2-deoxy-D-galactitol (XIV). Levene (56) cited the constants: m.p. 122°C; $\Delta T^{24}_D -21°$ (3.2, ethanol) and Wolfson (61) cited the constants: m.p. 121-123°C; $\Delta T^{21}_D -23°$ (1.8, ethanol). This compound was considered by the latter workers to be methyl hepta-0-4-0-(N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-L-
gulonate (VI) in which the C-1 atom of the hexosamine was joined in the glycosidic linkage with the C-4 atom of the uronic acid reduced fragment. The work of Meyer and Davidson designated chondrosine as a β-D-glucuronidochondrosamine in disagreement with the interpretation of Levene and of Wolf from and coworkers, the latter of which designated chondrosine as 4-O-(2-amino-2-deoxy-β(?)D-galactopyranosyl)-D-glucuronic acid.

These workers have presented evidence that chondrosine methyl ester hydrochloride on reduction with sodium borohydride followed by acetylation (preferential on N) yielded 3(?)O-(β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-galactitol. They have indicated also that reduction of chondrosine with sodium borohydride followed by esterification and acetylation yielded crystalline 3(?)O-(methyl hepta-O-acetyl-β-D-glucopyranosyluronate)-2-deoxy-2-acetamido-D-galactitol. The hydrolysis of the amorphous N-acetyl derivative with β-D-glucosidase indicated a β-D configuration of the disaccharide. The proposed reaction sequence of Davidson and Meyer is as follows.
The results of the findings of these two groups of workers are not inconsistent with each other. The compound designated by Wolf from and coworkers as methyl 4-O-(hepta-O-acetyl-N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-L-gulonate (VI) was adequately described by these workers as it was by Meyer and Davidson in their designation of the compound as methyl hepta-O-acetyl-3(?)O-(β-D-glucopyranosyl uronate)-2-acetamido-2-deoxy-D-galactitol (XIV). The compound designated by Wolf from and coworkers as 4-O-(N-acetyl-2-amino-2-deoxy-D-galactopyranosyl)-L-gluconamide (VII Madison's IV, ref. 60) was found to consume 2 moles of periodate nearly instantaneously with the immediate formation of 1 mole of formaldehyde and 1 mole of formic acid. This is proof of the sequence —CHOH-CHOH-CH₂OH. The proposed formulas of Meyer and of Wolf from both contain this sequence. Madison obtained a crystalline product on employing 2 moles of periodate. Further consumption of periodate on XVI (Meyer's monosaccharide order) was found by Madison to be slow and was in harmony with a glycol group adjacent to an amide group. Such a glycol group was known to be attacked slowly by periodate (68).


However, the Meyer formula likewise contains such a glycol
grouping. Madison considered the slow oxidation as "over-oxidation" and extrapolated back to a 3 mole total consumption; however, a slow uptake to 4 without significant over-oxidation would conform the periodate data to Meyer's formula. On this basis, Madison's crystalline periodate oxidation product would have the structure XVIII, which is a new type of disaccharide formed from a D-glucuronic acid unit and a 3-amino-3-deoxy-L-threotetrose entity, both of which have had their acidic and basic groups neutralized by conversion to amides.

![Chemical structures](image-url)
One other point should be made at this time. In the paper of Levene (56), published after his death in 1941, the assumption was made that the reducing portion of chondrosine was in the D-glucuronic acid portion of the molecule and thus the carbonyl function of the D-chondrosamine was bound in the glycosidic linkage. This assumption was based upon the findings of Levene and LaForge (69) in their study of the oxidation of chondrosine with nitric acid followed by alkaline hydrolysis and isolation of the saccharic acid. In the light of modern knowledge, this proof that the uronic acid was free in chondrosine can be considered as entirely inadequate.

Very recently Davidson and Meyer (70) have characterized chondrosine as glucuronidochondrosamine. Chondrosine methyl ester hydrochloride was deaminated by ninhydrin and converted to 2(β-D-glucopyranosyl uronic acid)-D-lyxose. This compound was re-esterified and reduced with sodium borohydride in borate buffer to 2-O-(β-D-glucopyranosyl)-D-lyxitol, characterized as...
the crystalline octaacetate. This compound was deacetylated and submitted to periodate oxidation. The disaccharide consumed four moles of periodate with the production of two moles of formic acid and one mole of formaldehyde. These data required a $1 \rightarrow 2$ linkage and therefore a $1 \rightarrow 3$ linkage in chondrosine.

Other than the papers just reviewed the studies of chondroitinsulfate since 1950 have been of a more generalized nature. These will be reviewed briefly in the discussion to follow.

The crystallization of chondroitinsulfuric acid has been reported (71). Einbinder and Schubert made an

(71) J. Einbinder and M. Schubert, J. Biol. Chem. 185, 725 (1950); ibid, 191, 591 (1951).

exhaustive study of methods of extracting chondroitinsulfate from cartilage. These workers extracted chondroitinsulfate from bovine tracheae by means of aqueous potassium chloride. They compared the purity of this material, after purification, with that obtained by other extraction techniques. In a continued study of this material these workers reported a technique by which the potassium salt was converted to the calcium salt and separated into three fractions by the careful addition of ethanol to a dilute aqueous acetic acid solution of this material. By means of a fractional precipitation these workers
reported the isolation of a small amorphous head fraction, a crystalline mid-fraction and a semi-crystalline tail fraction. After subsequent drying these workers reported optical rotations for these three fractions to be $\alpha^{23}\mathrm{D} = -29.6^\circ$, $-24.7^\circ$ and $-21.8^\circ$, respectively ($c 2.5$, $\mathrm{H}_2\mathrm{O}$). These workers published a detailed analytical evaluation of these fractions as compared to potassium chondroitinsulfate prepared by their procedure. The hexosamine, hexuronic acid and elemental analysis of the above cited fractions showed no great differences between the fractions. However, the analytical values for these fractions did not agree too well with the calculated values proposed by these workers. Two other significant points were found in these results. These workers reported that on drying the calcium salts and the potassium salt of chondroitinsulfate at $100^\circ$ in vacuum over phosphoric anhydride for 8-10 hours, water corresponding to a pentahydrate (Ca: $\mathrm{H}_2\mathrm{O} = 1:5$) for the calcium salts and a dihydrate (K: $\mathrm{H}_2\mathrm{O} = 1:2$) for the potassium salt was lost. Further, these workers reported that chondroitinsulfate could not be crystallized unless the material was mixed with aqueous suspension of kaolin seven times and recovered. In their involved purification procedure, these workers gave no reason for this required step.

In the first of a series of papers concerned with
their study of chondroitinsulfuric acid, Dorfman (72)


reported that hyaluronidase prepared from bacteria and leeches did not hydrolyze chondroitinsulfate. In the second publication (73) this laboratory reported that


chondroitinsulfuric acid, prepared by the procedure of Meyer, Odier and Siegrist (57) was enzymically hydrolyzed by testicular hyaluronidase. In this paper the authors published an interesting method of following the rate of hydrolysis. They made use of the turbidity produced by the interaction of acidified bovine albumin and chondroitinsulfate in aqueous solution at pH 4.00. They found a linear relationship between the chondroitinsulfate concentration and the turbidity of the solution.

In their next publication these workers published an interesting purification procedure for isolating chondroitinsulfate from beef nasal septa (74). They com-


pared the purity of the chondroitinsulfate with that
prepared by the Einbinder and Schubert (70) procedure, alkali extracted chondroitinsulfate and potassium chloride extracted material. These workers extracted the crude chondroitinsulfate from the beef septa, clarified the extract, dialyzed the material and precipitated the product by the addition of the complexing agent CO(NH$_3$)$_6$Cl$_3$ complex. Further purification by customary precipitation methods yielded 5-6 g. of purified sodium chondroitin-sulfate from 100 g. of nasal septa. In the presentation of their analytical findings these workers reported water loss in chondroitinsulfate, after drying in vacuo for 60 hours at 78°, to be from 5-8 percent for various samples analyzed. Furthermore, these workers reported that drying sodium chondroitinsulfate for several days in vacuo over phosphoric anhydride resulted in the material still retaining about 10 per cent water. The analyses of their preparations are shown in Table II. That water of hydration was still present in their dried samples was very apparent from these data.

These workers conducted molecular weight studies of the chondroitinsulfate prepared by their procedure. They found the material to be polydisperse in molecular size. They found the material prepared by their procedure to be of higher molecular weight than that prepared by potassium chloride extraction of stored wet cartilage. Osmotic pressure measurements indicated a molecular weight
Table II

Chemical Analyses of Chondroitinsulfuric Acid Preparations (74)

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Loss on drying</th>
<th>N, %</th>
<th>H(_4)AsO(_4), %</th>
<th>S as SO(_4), %</th>
<th>AsH(_2)(Na(_2)SO(_4)), %</th>
<th>CO(_2)Na, mg./g.</th>
<th>Fraction of Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS(_3)(^{(f)})</td>
<td>6.7</td>
<td>2.9</td>
<td>7.4</td>
<td>6.1</td>
<td>25.7</td>
<td>2.00</td>
<td>0.936</td>
</tr>
<tr>
<td>CS(_5)(^{(b)})</td>
<td>5.7</td>
<td>2.9</td>
<td>7.7</td>
<td>5.8</td>
<td>25.3</td>
<td>1.83</td>
<td>0.906</td>
</tr>
<tr>
<td>4-1068(^{(c)})</td>
<td>8.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS(_4)(^{(d)})</td>
<td>6.4</td>
<td>2.9</td>
<td>7.7</td>
<td>5.8</td>
<td>24.2</td>
<td>1.97</td>
<td>0.914</td>
</tr>
<tr>
<td>CS(_1)(^{(e)})</td>
<td>7.8</td>
<td>2.8</td>
<td>7.7</td>
<td>5.5</td>
<td>23.8</td>
<td>1.90</td>
<td>0.890</td>
</tr>
<tr>
<td>CS(_13)(^{(e)})</td>
<td>5.0</td>
<td>2.9</td>
<td>7.9</td>
<td>6.0</td>
<td>25.6</td>
<td>1.85</td>
<td>0.925</td>
</tr>
<tr>
<td>Calculated values</td>
<td>0</td>
<td>2.78</td>
<td>8.55</td>
<td>6.37</td>
<td>28.25</td>
<td>1.98</td>
<td>1.000</td>
</tr>
</tbody>
</table>

(a) Loss at 78°, in vacuo over P\(_2\)O\(_5\) for 60 hr. All other analytical values in this table have been corrected for this loss.
(b) Present extraction technique and purification.
(c) Einbinder and Schubert sample.
(d) Potassium chloride extraction technique from stored wet cartilage.
(e) Alkali-extracted material.
(f) For \(G\)\(_{14}\)H\(_{19}\)O\(_{14}\)NSNa\(_2\) repeating period.
of 43,300 for the chondroitinsulfuric acid prepared by this procedure. Intrinsic viscosity measurements were made with the various chondroitinsulfuric acid samples and molecular weights of 13,800 - 43,300 were obtained for the various preparations. These workers also stated that the shape of the chondroitinsulfate molecule in solution was dependent upon the ionic environment.

In their next publication (75) this laboratory conducted a very thorough study of the molecular weight of chondroitinsulfuric acid from the physical chemical point of view. These workers made extensive viscosity studies of the material and noted the effects of electrolytes in solution on the viscosity of the material. Potentiometric titrations were conducted in aqueous solutions of the polymer in determining the hydrogen ion activities in such a solution. In this procedure known amounts of hydrochloric acid were added and titrated potentiometrically with base. By this and similar titrations the capacity of chondroitinsulfuric acid, in aqueous solution, to bind various cations was determined quantitatively. These workers concluded that chondroitinsulfuric acid was a linear polyelectrolyte in aqueous solution. Other workers had considered it to be similar.
to an ion-exchange resin (46). These workers submitted support for these earlier findings and noted further that striking changes in the configuration of the chondroitin-sulfate molecule may result in many of these processes.

In a publication in 1951, Meyer and Rapport (76) conducted a study of the mucopolysaccharides in the ground substance of connective tissue. After extracting the polysaccharides with dilute alkali followed by the well-known amyl alcohol purification treatment and amylase digestion they fractionally precipitated the materials from a dilute calcium acetate-acetic acid solution by the addition of alcohol. These workers separated the material with five fractions from which they obtained two different chondroitinsulfate materials which they designated chondroitinsulfate A and B, respectively.

Chondroitinsulfate A had an $[\alpha]_D^2 -30^\circ$ and was hydrolyzed by testicular hyaluronidase but not by pneumococcul hyaluronidase. Chondroitinsulfate B had the same composition as A but a specific rotation of $-50^\circ$ and was resistant to hydrolysis by both testicular hyaluronidase and pneumococcul hyaluronidase. The workers considered this material the same as that isolated earlier from pig skin (43).
Wolf from and Neely (77) have confirmed the identification of the uronic acid component of chondroitinsulfuric acid. These workers applied the oxidative hydrolysis procedure utilized in the identification of the uronic acid component present in heparin and mucosulfuric acid (18,78).

Purified barium chondroitinsulfate was oxidized with bromine in concentrated sulfuric acid for six days at 30°C. From the reaction mixture, after bromine removal and neutralization, was isolated potassium acid D-glucarate (D-glucosaccharate). X-Ray diffraction studies and optical activity measurements in neutral aqueous solution, $\lambda_\alpha^2D +10^\circ$, positively identified the crystalline compound obtained in 50 mg. yield from 500 mg. of chondroitinsulfuric acid.

This work extended the early findings of Levene and Jacobs (51) in their isolation of an uncharacterized (other than by analysis) silver salt of a hexaric acid after oxidative hydrolysis of chondroitinsulfate with bromine in hydrobromic acid. These workers called
chondroitinsulfate "glycothionic acid" at that early
date. Stacey had confirmed the presence of uronic acid
in chondroitinsulfate by methylation techniques (52).

Davidson and Meyer (79) have announced, on frac-

tionation of corneal polysaccharides, the identification
of a new chondroitinsulfuric acid with a sulfur content
of 2.1 per cent. This material was shown to contain
chondrosamine, uronic acid and was split by both
pneumococcal and testicular hyaluronidase. On hydroly-
sis, chondrosine, the disaccharide repeating unit of
ordinary chondroitinsulfate, was obtained in 75 per cent
yield. This material was stated to be very similar to
ordinary chondroitinsulfate except for the low sulfur
content.

Herein has been presented a brief survey of some
of the more important findings that were related to the
structure of pure chondroitinsulfuric acid. This was
considered necessary for the correlation of the general
chemical similarities of this mucopolysaccharide and
heparin. Due to these similarities it was considered
possible to convert chondroitinsulfuric acid to a
material with the physiological properties of heparin.

(79) E. A. Davidson and Karl Meyer, Abstracts
C. The Sulfation of Polysaccharides

Demole and Reinert (80) and Fischer (81) were the first to bring attention to the fact that blood anticoagulants such as hirudine and heparin contained the sulfate group. However, it was the work of Bergström (82) that aroused an interest in the preparation of sulfated polysaccharides. In his study of the structure of heparin, Bergström found that the polysaccharide contained ester-bound sulfate corresponding to 7-8 per cent sulfur. Otherwise, the material appeared to be very similar in chemical composition to chondroitinsulfuric acid. On the basis of these findings, Bergström sulfated a number of mono-, di- and poly-saccharides by the chlorosulfonic acid-pyridine method of Gebauer-Füllnegg and associates (83).

Bergström prepared sulfate esters of chitin, cellulose, pectin, starch, chondroitinsulfuric acid, glycogen, gum
arabic and nucleic acid. When tested for anticoagulant activity the sulfated chondroitin sulfate was found to be the most active. The sulfated simple sugars were inactive. The publication of these results led to the sulfation of a number of polysaccharides.

Sulfated cellulose has been prepared by a number of other workers (82, 84-91). Sulfated starch has been prepared (84, 88-92) as well as inulin (93), xylan (85), glycogen (77, 85), alginic acid (94, 95), dextran (96, 97),

(84) P. Glasson, J. prakt. Chem., 27, 12, 1 (1879).
(85) W. Traube, B. Blazer and E. Ludeman, Ber., 62, 603 (1930).
(86) I/S Solusol, Danish Patent 65269 (1946).
(96) A. Gronwall, B. Ingelmann and H. Mosimann, Upsala Lakorförening Förh., 50, 397 (1945); Chimie & Industrie, 55, 206 (1946).
chitin (82,88), chitosan (97-99), polyvinyl alcohol (89),

pectin (82,88) and levoglucosan (88). Chondroitin sulfate has been sulfated (82,88).

Karrer, Koening and Usteri (88) realized that many of the sulfated polysaccharides were very toxic in the human system. At this time the relation between the toxicity and constitution of these materials was not understood. These workers sulfated chondroitin sulfuric acid, cellulose and pectin and noted that the materials were about one-fourth to one-sixth as active as heparin in preventing the clotting of blood. These workers considered the high toxicity of sulfated cellulose to be due to the easy saponification of the ester bonds in solution followed by precipitation of the material. They therefore prepared glycolic acid derivatives and 2-hydroxyethane sodio sulfate derivatives of cellulose prior to sulfation of the material in the cold. Products obtained in these experiments were less toxic than sulfated cellulose but were also less active as anticoagulants.
Husemann and coworkers (89) were among the first to find some definite relationship between the toxicity of sulfated polysaccharides and their molecular weights. These workers prepared a number of sulfated polysaccharides and studied sulfated cellulose, in particular. They prepared a series of sulfate esters of varying molecular weights and studied the relative toxicities and anticoagulant activities of these different molecular weight materials. They found a definite inverse relationship between the molecular weight and the toxicity of these sulfate derivatives when tested in vivo. They found the same relationship with varying molecular weight in sulfated polyvinyl alcohols.

Richetts and Walton (97) prepared dextran sulfates of different molecular weights and found a definite critical molecular weight for this material below which it was not excessively toxic and had much the action of heparin. Ricketts (98) also prepared sulfated chitosan and studied its anticoagulant activity. He found that this material, prepared by the familiar chlorosulfonic acid-pyridine procedure, had a sulfur content of 15.1 per cent and approximately 11 per cent of the anticoagulant activity of heparin.

Walton (100) found that fibrinogen formed a precipi—

tate with heparin or dextran sulfate, of molecular weight 7000-8000, 47,000, 128,000 and 458,000 below its isoelectric point. He also found that fibrinogen formed a precipitate above its isoelectric point only with dextran sulfate of molecular weight 47,000 or greater. This worker noted that the precipitates formed on either side of the isoelectric point of fibrinogen were different. He noted that the precipitate above the isoelectric point was an easily dissociated complex dependent primarily upon the molecular weight of the dextran sulfate and not the content of acidic groups. This worker stated that this phenomenon was related to that encountered in vivo with high molecular weight sulfated polysaccharides.

Meyer, Piroue and Odier (101) made a detailed study of the sulfation of chondroitinsulfuric acid prepared from nasal septa and bovine trachea. These workers utilized starting chondroitinsulfate of a molecular weight of about 30,000.

These workers utilized variations of two well known sulfation procedures in their experiments. They employed the pyridine-chlorosulfonic procedure of Gebauer-Füllemeg (83) and the sulfuric anhydride procedure suggested and utilized by other workers (102,103).
After isolation of their products these workers gave a complete evaluation of each method. They determined the molecular weights of some preparations as well as anticoagulant activity studies. Time of clotting studies and other evaluations were made. A complete evaluation of their findings of chemical nature are given in the following Table III prepared by these workers for the sulfated chondroitinsulfuric acid and other sulfated polysaccharides.

Viscosity studies of sulfated chondroitin sulfate prepared by the $\text{SO}_2-\text{SO}_3-\text{HCl}$ and $\text{SO}_2-\text{SO}_3$ procedures were conducted and these materials were found to have molecular weights of 26,000 and 28,000, respectively, specific and optical rotations of $-20^\circ$ and $-21^\circ$, respectively, at 20$^\circ$. The starting chondroitin sulfate had a molecular weight of 30,000 and an optical rotation of $[\alpha]_D^{20} -30^\circ$.

An "in vivo" anticoagulant activity determination was made of the above two materials on rabbits according to the Soulier (104) modification of the Waugh and

## Table III

Resume of Results (101)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>% Water</th>
<th>Sulfation method</th>
<th>Temp °C</th>
<th>Time hr.</th>
<th>Degradation</th>
<th>Yield %</th>
<th>Sulfur %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chondroit-insulfate</td>
<td>19</td>
<td>$SO_2-SO_3$</td>
<td>-20</td>
<td>6</td>
<td>very weak</td>
<td>86</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>$SO_2-SO_3\cdot HCl$</td>
<td>-20</td>
<td>6</td>
<td>weak</td>
<td>90</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>pyridine-$SO_3$</td>
<td>60</td>
<td>6</td>
<td>weak</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>pyridine-$SO_3$</td>
<td>20</td>
<td>17</td>
<td>very weak</td>
<td>75</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Dioxane-$SO_3$</td>
<td>20</td>
<td>25</td>
<td>very weak</td>
<td>90</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>$SO_3\cdot HCl$</td>
<td>-12</td>
<td>17</td>
<td>very strong</td>
<td>55</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Pyridine-$SO_3$</td>
<td>60</td>
<td>6</td>
<td>strong</td>
<td>70</td>
<td>14</td>
</tr>
<tr>
<td>Galactomannan</td>
<td>7</td>
<td>$SO_2-SO_3$</td>
<td>-20</td>
<td>6</td>
<td>strong</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>Lichenin</td>
<td>11</td>
<td>$SO_2-SO_3$</td>
<td>-20</td>
<td>6</td>
<td>moderate</td>
<td>60</td>
<td>15</td>
</tr>
</tbody>
</table>
Ruddick (105) procedure. These materials were found to have activities of 50-60 and 50 per cent respectively of standard Connaught heparin. Time of clotting studies indicated that sulfated chondroitinsulfate compared favorably with heparin in that about twice as much of the former was required to prolong clotting of blood the same length of time when a given amount of the latter was employed. In conclusion, it should be stated that these workers considered the sulfuric anhydride superior in several respects to the pyridine-chlorosulfonic acid procedure.

Doczi, Fischman and King (106) have sulfated chito-
<table>
<thead>
<tr>
<th>Sample</th>
<th>Sulfur, %</th>
<th>Moles-SO$_3$Na/repeating unit</th>
<th>Activity I.U./mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>16.17</td>
<td>1.68</td>
<td>13</td>
</tr>
<tr>
<td>B</td>
<td>16.74</td>
<td>1.80</td>
<td>59</td>
</tr>
<tr>
<td>C</td>
<td>14.28</td>
<td>1.32</td>
<td>11</td>
</tr>
<tr>
<td>D</td>
<td>14.27</td>
<td>1.32</td>
<td>57</td>
</tr>
<tr>
<td>E</td>
<td>15.99</td>
<td>1.64</td>
<td>57</td>
</tr>
</tbody>
</table>
Recently, results from the sulfation of N-deacetylated chitin and partially N-deacetylated chondroitin-sulfuric acid were reported from this laboratory (107).


Both materials were sulfated by the sulfation procedure utilizing pyridine and chlorosulfonic acid.

N-Deacetylated chitin (108) was sulfated for 1 hour at 100°C. and yielded a product which had an anticoagulant activity of 56 I.U./mg. and an animal (mouse intravenous) toxicity approximately twice that of heparin. On analysis this material was found to contain approximately one O-sulfate and two N-sulfate groups per disaccharide repeating unit. This material exhibited a behavior characteristic of an acid-labile N-sulfate group on submission to the Van Slyke amino acid assay as found with heparin (23,29).

Cushing, Davis, Krotovil and MacCorquodale (109)

have published the results of their findings with regard to the sulfation of chitin with chlorosulfonic acid in a dichloroethane solvent. These workers prepared a series of different molecular weight chitin sulfates and determined the anticoagulant potency, per cent sulfur, per cent nitrogen, and viscosity of each sample. In each case an 8.0 g. sample of chitin was sulfated to give the yields shown in the following Table V after due purification of the product in each case. Molecular weight determinations were made using an Ostwald-Fenske viscometer.

These workers made some rather interesting observations concerning this material. Firstly, they intentionally degraded the chitin during sulfation, unlike previous workers, in order to minimize toxicity effects. These workers found that unbuffered solutions of sodium chitin sulfate fell in pH and that loosely sealed dry samples of this material lost much of their potency. They found that raising the pH of such solution to 11.0-11.5 brought about stabilization of the material. Furthermore, they found that peroxide treatment of chitin sulfate solution removed much of the undesirable color. They found no significant peaks in the adsorption spectra of this material. Molecular weights (by light scattering) of chitin sulfate so prepared were found to be between 12,100 and 17,100 for various preparations.
<table>
<thead>
<tr>
<th>Pro- Yield</th>
<th>S, %</th>
<th>N, %</th>
<th>In Vitro Potency, I.U./mg.</th>
<th>Hsp/6, Cl, H2O</th>
<th>Hsp/6, Cl</th>
<th>Ave. mol. wt.</th>
<th>Ref. 725-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11-12</td>
<td>13.2-14.5</td>
<td>2.5-3.2</td>
<td>26-34</td>
<td>0.30-0.38</td>
<td>0.14-0.16</td>
<td>17,100</td>
</tr>
<tr>
<td>2</td>
<td>10.5-11.5</td>
<td>14.5-15.0</td>
<td>26-30</td>
<td>0.26-0.30</td>
<td>12,100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10.5-11.0</td>
<td>15-15.5</td>
<td>22-26</td>
<td>0.25-0.30</td>
<td>14,800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8.9</td>
<td>13.9-14.5</td>
<td>24-27</td>
<td>0.35-0.40</td>
<td>14,700</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table V
Sodium Chitin Sulfate (109)
Data pertinent to their materials are given in Table V.

At this time it is considered worthwhile to mention two patents which have been issued for the preparation of sulfated polysaccharides of the type under discussion.

Hadidan (110) was issued a patent for the sulfation of hyaluronic acid under a variety of conditions. Sulfating agents included chlorosulfonic acid, chlorosulfonic acid-pyridine, chlorosulfonic acid in formamide solvent and fuming sulfuric acid under a variety of experimental conditions. This worker claimed a variety of sulfated hyaluronic acid products of different viscosities and sulfur contents. Various anticoagulant activities are claimed for different preparations.

Pulver (111), in his sulfation of chondroitinsulfuric acid, made use of formamide as the solvent. This worker employed chlorosulfonic acid as the sulfating agent. It was claimed that this method produced a less toxic sulfation product than previous procedures.
III. EXPERIMENTAL

A. Purification of Sodium Chondroitinsulfate and Preparation of its Salts

1. The Preparation of Purified Sodium Chondroitinsulfate:- An amount of 50 grams of crude sodium chondroitinsulfate (Wilson Laboratories, Inc., Chicago, Illinois) was dissolved in 500 ml. of distilled water. Solution was obtained by adding water slowly to the tan powder, with grinding, to yield a thick syrup. This syrup was diluted slowly with distilled water until a solvent volume of 500 ml. was obtained. This dark, cloudy solution was stirred overnight and centrifuged to remove much of the tan suspended material. The solution was then filtered through a coarse fritted glass funnel.

A chromatographic column (45 x 225 mm.) was packed with dry Magnesol-Celite (5:1) under vacuum to give a rather tight column. While the vacuum was still being applied, 500 ml. of distilled water was passed through the column. The vacuum was released when the last of the water started down the column. The volume of the water in the receiving flask was measured. Vacuum was again applied and the chondroitinsulfate solution started down the column. The calculated amount of forerun distilled water was collected and discarded with the above water. The chondroitinsulfate solution was passed down
the column at a moderate rate. A tan band appeared on the column. It was noted that this band was moving more slowly than the solution itself but became very broad before all of the solution had been chromatographed. The collection of eluent was stopped when the top of the column started to become dry. The column was not rinsed until the eluent was removed from the receiving flask. The lemon yellow eluent was usually turbid at this point in the purification process. The usual methods of clarification by filtration and centrifugation were attempted. However, they were unsuccessful. Filtration through an asbestos mat completely cleared the solution. This filter was prepared by placing a layer of acid-washed asbestos on Whatman No. I filter paper in a funnel (Büchner). This layer was washed with suction, with at least one liter of distilled water to remove all of the same asbestos particles. A circle of Whatman No. I filter paper was placed over the asbestos layer and the chondroitin sulfate solution passed through to yield a sparkling clear lemon yellow solution.

The neutral solution now had a volume of about 400 ml. To this stirred solution was added 800 ml. of c.p. methyl alcohol followed by 700 ml. of 95 per cent ethyl alcohol. A white voluminous precipitate usually resulted. However, it was found that the addition of about 5 ml. of saturated aqueous sodium chloride solution at the point
of first turbidity greatly facilitated precipitation. The voluminous white precipitate was allowed to settle and the almost colorless supernatant liquid removed by filtration. The resultant white material was washed well with 95 per cent ethyl alcohol and absolute ethyl alcohol and redissolved in an amount of 400 ml. of distilled water and the precipitation repeated. The product was washed well as described and then washed with absolute ethyl ether and immediately dried in water pump vacuo over phosphoric anhydride at room temperature. Since the material was washed in a Büchner funnel under suction the evaporation of ether cooled the solid material and thus caused water vapor to condense on the surface. This often led to the siruping of the product if not dried immediately. The final product was a pure white powder. This was further purified by dialysis (using a cellulose membrane of porosity such that it retained particles of molecular weight larger than 8000) of an aqueous solution against distilled water for two days followed by lyophilization to remove the water. The final product gave a negative ninhydrin test, a negative Benedict test and a positive test for bound sulfate. As described later, this material, on acetylation, gave a product almost identical with that obtained from the acetylation of sodium chondroitinsulfate prepared by the acetic acid purification procedure. Yield 23 grams,
This material gave a positive test for hexosamine and uronic acid. All samples analyzed by the departmental analyst were dried at 78 °C over phosphoric anhydride for at least 60 hours at oil pump vacuum. Samples analyzed by Huffman Microanalytical Laboratories were dried to constant weight, at oil pump vacuum over phosphoric anhydride.

Analysis: Calculated for C_{14}H_{19}NO_{11}Na(SO_{3}Na^+_{2}H_{2}O)_{0.745}. C, 33.45; H, 4.4; N, 2.78; S, 4.72; Na, 7.96.

Found: C, 33.20 (d), 33.41 (d); H, 4.53 (d), 4.47 (d); N, 2.79 (H); S, 4.72 (H); Na, 7.89 (d), 7.86 (d).

The Magnesol-Celite column used in this purification procedure was rinsed with 250 ml. of distilled water to yield a very dark brown solution. The product was collected by precipitation with methanol to yield a tan powder. This powder was dissolved in 250 ml. of distilled water and precipitated by the addition of 500 ml. of grain alcohol to give a turbid solution. Final precipitation was obtained by the addition of 500 ml. of methanol and 5 ml. of saturated aqueous sodium chloride solution. The tan precipitate was collected by centrifugation and the material redissolved in 250 ml. of distilled water and precipitated as before. The final

(d) Departmental analyst, W. H. Deebel.
(H) Huffman Microanalytical Laboratories, Wheatridge, Colorado.
product was washed well with 95 per cent ethyl alcohol and absolute ether and dried in water pump vacuo over phosphoric anhydride at room temperature to yield a tan powder. The final yield was 10.9 grams.

This tan material was found to be very reducing to Benedict's solution and it was found to give a positive ninhydrin test for free amino groups. An aqueous solution of this material was found to be too dark for a satisfactory optical rotation determination. The analytical data on this material were calculated on the basis of hydrate formation and were found to be satisfactory with the purified sodium chondroitinsulfate prepared by this procedure.

**Analysis (a):** Calculated for $\text{C}_{14}\text{H}_{19}\text{NO}_{11}\text{Na}(\text{SO}_3\text{Na} \cdot 2\text{H}_2\text{O})$

- C, 33.45; H, 4.4; N, 2.78; S, 4.72; Na, 7.96.
- Found: C, 32.00 (d), 32.23 (d); H, 4.99 (d), 4.71 (d); N, 3.00 (d), 2.79 (d); Na, 8.98 (d), 8.42 (d).

2. **The Preparation of Sodium Acid Chondroitinsulfate**

(a) **Chromatographic Procedure:** An amount of 50 grams of crude sodium chondroitinsulfate (Wilson Laboratories, Inc. Chicago, Illinois) was chromatographically purified as previously described to give 400 ml. of clear lemon yellow solution. The chondroitinsulfate was caused to

(a) Dried as previously stated.
(d) Departmental analyst.
precipitate by the slow addition of 3 liters of glacial acetic acid with stirring. The white gelatinous precipitate was allowed to settle for 4 hours and collected in a sintered glass funnel. This precipitate was washed well with 95 per cent ethyl alcohol. This precipitate was dissolved in 400 ml. of distilled water and precipitated by slow addition of 300 ml. of 95 per cent ethyl alcohol with stirring. The addition of about 5 ml. of saturated aqueous sodium chloride at the point of turbidity aided the precipitation but was not always necessary. The voluminous white precipitate was washed well with 95 per cent ethyl alcohol, absolute ethyl alcohol and absolute ether and redissolved in 400 ml. of distilled water and the precipitation and washing repeated. The pure white powder was dried at room temperature in water pump vacuo over phosphoric anhydride. In some runs of this procedure further purification was affected by dialysis of an aqueous solution followed by lyophilization (85 per cent yield for the step).

The final product, without dialysis, was a white powder which gave a negative ninhydrin test, negative Benedict test and a positive test for bound sulfate. Analysis of the material showed it to be slightly low in sulfur content and to contain about one molecule of water of hydration for each sulfate group present. This may have been due to incomplete drying prior to
to analysis although the material was dried in oil pump vacuo over phosphoric anhydride at 78°C. for at least 60 hours, or as specified in Sec. A, Part I, of Experimental. Yield 22 grams; \(\mu_0^2\)-D -15.42° (c 1.46, H\(_2\)O).

Analysis: Calculated for C\(_{14}\)H\(_{20}\)NO\(_{11}\)(SO\(_3\)Na\(\cdot\)H\(_2\)O)\(_{0.816}\):
C; 35.3; H, 4.54; N, 2.93; S, 5.48; Na, 3.94%. Found:
C, 35.00 (d), 34.74 (d); H, 4.70 (d), 4.87 (d);
N, 2.99 (H); S, 5.43 (H); Na, 4.04 (d), 4.06%(d).

(b) The Glacial Acetic Acid Procedure:—As a final proof of the inadequacy of the standard purification procedure (R. K. Madison) for the purification of highly impure or degraded chondroitin sulfate a quantity of impure chondroitin sulfate was purified by glacial acetic acid precipitation without making use of the chromatographic purification step.

An amount of 50 grams of crude sodium chondroitin-sulfate (Wilson Laboratories, Inc., Chicago, Illinois) was dissolved in 400 ml. of distilled water, clarified by filtration through a thin Magnesol mat and precipitated by the slow addition of 3 liters of glacial acetic acid with stirring. The tan precipitate was collected in a sintered glass funnel and washed well with 95 percent ethanol. This material was dissolved in 400 ml.

(d) Departmental analyst.
(H) Huffman Microanalytical Laboratories, Wheatridge, Colorado.
of distilled water and precipitated by the slow addition of 800 ml. of c.p. methyl alcohol followed by 700 ml. of 95 per cent ethyl alcohol with stirring. The voluminous precipitate was collected by filtration and washed well with 95 per cent ethanol, absolute ethyl alcohol and absolute ether. The material was redissolved in distilled water and reprecipitated and washed as before.

This tan powder was immediately dried at room temperature in water pump vacuo over phosphoric anhydride. Yield 37.4 grams. This material gave a positive Benedict's test and a positive test for the free amino group (ninhydrin). However, it gave a positive hexosamine test.

The analytical calculations were based on hydrate formation as was found with pure sodium acid chondroitin sulfate prepared by the chromatographic modification. The optical rotation was $[\alpha]_D^{21} = -20.34^\circ$ (c 1.46, H$_2$O).

Analysis (a): Calculated for C$_{14}$H$_{20}$O$_{11}$S$_3$Na$^+$H$_2$O$_{0.816}$: C, 35.3; H, 4.54; N, 2.93; S, 5.48; Na, 3.94.

Found: C, 34.11 (d), 34.34 (d); H, 4.43 (d), 4.90 (d); N, 3.52 (H); S, 5.59 (H); Na, 5.8 (d), 5.9 (d).

(a) Samples dried as specified in III-A-1
(d) Departmental analyst
(H) Huffman Microanalytical Laboratories, Wheatridge, Colorado.
3. The Preparation of Calcium Chondroitin Sulfate:

An amount of 5.5 grams of sodium chondroitin sulfate (purified as in Sec. III-A-1) was dissolved in 100 ml. of distilled water and to this solution was added 1 drop of acetic acid and 2.5 grams of calcium chloride. Absolute ethyl alcohol was added to this solution with stirring. After the addition of 50 ml. of alcohol the solution became turbid. Addition was continued until a total of 100 ml. of alcohol was added. The white precipitate began to form and the solution was left overnight. Collection of this precipitate by centrifugation yielded a white granular powder. This was washed well with 75 per cent ethanol, absolute ethanol and ethyl ether and dried at room temperature in water pump vacuo over phosphoric anhydride. Yield 4.7 grams.

An amount of 3.7 grams of the above powder was dissolved in 207 ml. of distilled water. An amount of 7.14 ml. of glacial acetic acid and 0.476 grams of calcium chloride was added to this solution. An amount of 104 ml. of absolute ethyl alcohol was added to the above solution with stirring. The solution was then set aside for 2 days. At the end of this time a white-tan flocculent precipitate had settled from the solution. This was collected by careful centrifugation and dried at room temperature in water pump vacuo over phosphoric anhydride. Yield 0.164 grams.
To the above supernatant liquid was added absolute ethyl alcohol until the solution became turbid. This required about 35 ml. of alcohol. The solution was covered and left overnight. After standing one day the solution was turbid but no precipitate had formed. About 60 ml. of absolute ethyl alcohol was added to the solution with stirring and the mixture was set aside for one week. At the end of this time a pure white precipitate had formed. However, it was not crystalline. This material was washed well with grain alcohol, absolute alcohol and ethyl ether. The material was dissolved in 20 ml. of distilled water and absolute alcohol was added to the point of turbidity. This sample was set aside and labeled the mid-fraction.

To the remaining supernatant liquid of the above precipitation was added about 200 ml. of absolute ethyl alcohol with stirring. Addition was conducted over a period of 2 hours. After one week the solution, which had been set aside in a covered beaker was examined. A white precipitate had formed. This was collected by centrifugation, washed with grain alcohol, absolute alcohol and ethyl ether. The pure white material was dissolved in 20 ml. of distilled water and ethyl alcohol was added until the solution became turbid. This solution was labeled tail fraction and set aside one week.

At the end of one week the mid-fraction sample had
precipitated from solution as a white powder, the tail fraction had not. The addition of more alcohol to the latter solution brought about the precipitation of a white material.

Both precipitates were collected by centrifugation, washed with grain alcohol, absolute alcohol and ethyl ether and dried at room temperature in water pump vacuo over phosphoric anhydride. Yields for the mid and tail fractions were 1.5183 grams and 1.6391 grams respectively. These materials were pure white powder and gave positive tests for calcium, bond sulfate, hexosamine and uronic-acid. Optical rotations were determined and they were as follows: \( \gamma_{D}^{21} = -26.2^\circ \) and \( -22.9^\circ \) respectively. These two fractions were analyzed and the values for elemental analysis were as follows.

**TABLE VI**

Analysis of Calcium Chondroitinsulfate

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Carbon %</th>
<th>Hydrogen %</th>
<th>Calcium %</th>
<th>Nitrogen %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid</td>
<td>34.34,  5.21,</td>
<td>6.27,</td>
<td>2.74,</td>
<td></td>
</tr>
<tr>
<td>fraction</td>
<td>33.71</td>
<td>4.79</td>
<td>5.78</td>
<td>2.52</td>
</tr>
<tr>
<td>Tail</td>
<td>34.01,  4.46,</td>
<td>6.42,</td>
<td>2.56,</td>
<td></td>
</tr>
<tr>
<td>fraction</td>
<td>33.54</td>
<td>4.80</td>
<td>6.63</td>
<td>2.53</td>
</tr>
<tr>
<td>Calc. for</td>
<td>C(<em>{14})H(</em>{19})O(_{11})</td>
<td>33.75</td>
<td>4.44</td>
<td>6.99</td>
</tr>
<tr>
<td>(\frac{Ga(SO_{3}Ga)}{2})</td>
<td>0.745</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Samples were dried in oil pump vacuo over P\(_{2}O_{5}\) for 60 hr. prior to elemental analysis by department analyst.
B. N-Deacetylation of Chondroitin Sulfuric Acid

1. Attempted N-Deacetylation by Ester Exchange

with Sodium Methoxide in Methanol: - Deacetylation experiments were conducted on sodium chondroitin sulfate (purified as in III-A) according to the general procedure of Whistler and Jeanes (112). The procedure essentially was the attempted N-deacetylation using catalytic (or greater) amounts of sodium methoxide in absolute methanol on a quantity of sodium chondroitin sulfate suspended in this solvent. Methyl alcohol was distilled from the reaction mixture and replaced with fresh alcohol. The distillate was not analyzed but instead, the polymer was recovered and subjected to the modified Freudenberg analysis. Several experiments were conducted using this general procedure. Varying amounts of sodium methoxide were employed as well as varying amounts of polymer in varying degrees of activation. The products of the reaction usually gave a positive test for free amino but were only slightly N-deacetylated. A few representative experiments will be cited here.

An amount of 0.5 grams of sodium chondroitin sulfate was added to 400 ml. of absolute methanol and 10 ml. of 0.2 N sodium methoxide were added. The resulting
solution was refluxed one hour and the solvent distilled at the rate of about 20 drops a minute until only a small volume of solution remained. An amount of 40 ml. of absolute methanol was added and distilled from the reaction mixture. An additional 40 ml. of fresh alcohol was added and removed by distillation. The remaining solution was neutralized with dilute hydrochloric acid and the product, after solution in water, was precipitated with ethyl alcohol, washed well with ethyl alcohol and ethyl ether and dried at room temperature in water pump vacuo over phosphoric anhydride. A 0.1809 gram sample of this material, a tan powder, was analyzed for N-acetyl by the modified Freudenberg procedure described in Sec. B-2 (succeeding section). The acetyl content was 7.27 per cent which corresponded to a 15 per cent N-deacetylation.

The deacetylation reaction was repeated employing 50 ml. of methanol, 15 ml. of 0.2 N sodium methoxide and 1 gram of activated sodium chondroitinsulfate. The

*Sodium chondroitinsulfate was activated by the following procedure. The 1 gram sample was dissolved in about 25 ml. of distilled water and precipitated by the addition of 3 volumes of methanol. The resulting precipitate was collected by centrifugation and washed with several succeeding amounts of absolute methanol. This precipitate was washed by repeated mixing with fresh methanol and centrifuging until the material was suspended in dry absolute methanol. During this process the material never became "dry" but remained activated in that it was in a swollen state. Hereafter an activated sample shall signify one that is suspended in the appropriate medium.
methyl alcohol was distilled from the flask until only a small volume remained. An amount of 50 ml. of methyl alcohol was added and the suspension left in the distillation apparatus overnight. The sample was then neutralized and collected as previously described. This material was found to contain 7.40 per cent acetyl which corresponded to a 13.5 per cent deacetylation.

2. Acetyl Analysis:—The acetyl analysis used in this work was a modification of the original Freudenberg procedure (18,20). An amount of 0.175–0.200 grams of acetylated chondroitinsulfate, accurately weighed, was dissolved in 50 ml. of 20 per cent p-toluenesulfonic acid and the solution was refluxed one hour in the acetyl apparatus whose dimensions are accurately specified by Freudenberg and Harder (20). Water was then distilled from the solution until a 200 ml. sample was collected (in about 4 hours), fresh distilled water being slowly added to replace that distilled from the solution. The 200 ml. sample was titrated with 0.02 N sodium hydroxide. This process was repeated until consecutive 200 ml. aliquots yielded the same alkali titration. A blank was run using only 20 per cent p-toluenesulfonic acid.
This blank was subtracted from the titration values of the sample.

Acetyl analysis/purified sodium chondroitin sulfate, dried at room temperature in water pump vacuum over phosphoric anhydride for 48 hours prior to analysis, indicated an acetyl content of 8.07 per cent in the material. However, as found later, this analysis was possibly in error by 5 - 10 per cent due to the sample not being completely dry. Later work has shown that more drastic conditions of drying are required.

All acetyl analyses herein recorded were performed by this procedure.

3. Attempted N-Deacetylation by Cuprammonium Hydroxide: - An amount of 0.5 grams of sodium chondroitin sulfate (purified as in III-A) and 1 gram of cupric hydroxide were dissolved in 100 ml. of concentrated ammonium hydroxide (29 per cent \( \text{NH}_3 \) assay) to yield a clear blue solution. The solution was placed in a sealed flask and let stand for 3 weeks at room temperature. At the end of this time the polymer was precipitated by the addition of several volumes of 95 per cent ethanol. The precipitate was collected, redissolved in distilled water and reprecipitated by the addition of alcohol. The final product was collected by centrifugation and washed well with alcohol and ethyl ether to yield
a tan powder. This tan powder gave a very weak ninhydrin test for a free amino function. Since this test is very sensitive it was concluded that the product was not N-deacetylated.

4. Attempted N-Deacetylation with Dilute Sodium Hydroxide: An amount of 1.5 grams of sodium chondroitin-sulfate (purified as in III-A) was dissolved in 10.25 ml. of approximately 4 per cent aqueous sodium hydroxide which had been deaerated previously with nitrogen. The solution was agitated with a stream of nitrogen for 4 hours at room temperature and neutralized with N hydrochloric acid. The product was collected by precipitation with the addition of several volumes of methyl alcohol. The material was redissolved in distilled water, precipitated by the addition of alcohol, washed with alcohol and ethyl ether and the centrifuged precipitate dried at room temperature over phosphorous pentoxide at water pump vacuum. The final product was a white powder which gave a fair ninhydrin test for the free amino group. An N-acetyl analysis showed that this final product had an acetyl content of 7.87 per cent corresponding to a deacetylation of about 8 per cent.
5. The Deacetylation of Sodium Chondroitin Sulfate Using Concentrated Sodium Hydroxide.

(a) Procedure: - An amount of 65 ml. of approximately 50 per cent aqueous carbonate free sodium hydroxide was placed in an aeration flask and a stream of nitrogen (dry) passed through for 24 hours. An amount of 1 gram of sodium sulfite, dissolved in about 5 ml. of distilled water (warm), and 5 ml. of benzyl alcohol were added to the alkali solution and intimately mixed. An amount of 3 grams of sodium chondroitin sulfate (purified as in III-A) was dissolved in 10 ml. of distilled water and added to the above mixture. The top of the aeration flask was raised just enough to allow the sample to be poured in without disturbing the flow of nitrogen through the container. Just prior to sample addition the alkali solution was placed in an ice water bath. However, some local heating occurred and may have been beneficial for dissolution of the two solutions. After the sample addition the flask was closed and agitated by a vigorous stream of nitrogen for 1 hour, the solution being allowed to warm to room temperature and remain there for the remainder of the deacetylation. After 1 hour the rate of flow of nitrogen was decreased to a steady flow. The outlet tube of the aeration flask was fitted with a rather coarse capillary tube so as to minimize the inward flow of air into the flask (against the flow of nitrogen).
The reaction was continued for a total of 43 hours.

This alkaline chondroitinsulfate solution became tan colored during the reaction but not dark red. Some turbidity developed in the solution but the bulk of the sodium chondroitinsulfate was dissolved in the alkali. The turbidity was due, among other things, to some sodium sulfite precipitation and some precipitation of chondroitinsulfate. Therefore, the solution was saturated with respect to sodium sulfite and sodium chondroitinsulfate. This may have been an important factor in the preservation of the deacetylated material in that it may have been less soluble than the starting material. Such a precipitation should have tended to protect the material from alkaline hydrolysis due to the formation of two phases.

At the end of the reaction time the reaction vessel was cooled well in an ice bath and 200 ml. of c.p. methanol slowly added followed by 50 ml. of absolute ethyl alcohol. Just prior to this addition the nitrogen flow was increased to more effectively sweep the slightly opened vessel. The resulting solution, containing a voluminous white precipitate, was vigorously agitated with a stream of nitrogen, the flask being closed after the alcohol addition, for a period of one-half hour. Then the nitrogen flow was stopped, the flask was
completely sealed and the precipitate was allowed to settle in the flask. A pipette was inserted in the solution and the supernatant liquid drawn off. The flask was filled with fresh methanol, deaerated with nitrogen and the precipitate again allowed to settle. The supernatant liquid was drawn off as before and the alcohol addition and removal was repeated a third time. During this entire period the flask was suspended in an ice bath. After the third alcohol washing the solution was transferred quickly to centrifuge tubes, the tubes sealed with foil and the precipitate separated by dentrifugation. This precipitate was washed several times with methanol and ethanol while remaining in the tubes. The alcohols were cooled prior to use and the wash liquid was combined with the original alkaline solution. The product was dissolved in 100 ml. of distilled water and precipitated by the addition of 2 volumes of methanol until the solution followed by ethanol became turbid. About 10 ml. of saturated sodium chloride solution was added and the solution stirred vigorously. A white flocculent precipitate formed and more ethyl alcohol was added to give a total volume of about 600 ml. The precipitate was collected, redissolved in distilled water and precipitated as before. This process was repeated once more and the precipitate washed well with 95 per cent ethanol, absolute ethanol and absolute ether
respectively. The material, while still damp with ether was retained in centrifuge tubes. These were covered with filter paper and the ether slowly removed by evaporation under reduced pressure. Total yield after drying at room temperature over phosphorous pentoxide at water pump vacuum, was 2.5 grams of a white powder.

It was found that on dissolving the above product in a small amount of water (10-20 ml.) it could be separated into two fractions, a major water-soluble component and a minor component which was insoluble in the above amount of water. This insoluble material was collected by centrifugation and further studied. This material was found to be insoluble in aqueous solution about pH 7 but was soluble in water adjusted to pH 2-5 - 5-5 with dilute hydrochloric acid. The material was dissolved in thus acidified water and the solution made basic. This shift in pH caused the material to be precipitated as a white flocculent material. However, this material was found to be acid sensitive in that successive solution and precipitation as described above caused the yield to be successively lower with each operation. The material gave a good ninhydrin test for the free amino group and was found to have an acetyl content of 2.29 per cent. It was found also that the material was of low molecular weight in that a sample, dialyzed in a membrane, which held molecules of molecular weight
greater than 8,000, for 2 days against distilled water almost completely passed through the membrane. Dialysis was possible because the material was soluble in dilute aqueous solution. The total yield of this minor fraction, as first isolated by treatment of the 2.5 gram sample with a small amount of water and centrifugation, was 0.3 gram.

After removal of the above described minor fraction the major water soluble fraction was isolated in 2.1 gram yield by alcohol precipitation followed by washing with alcohol and ether and drying at room temperature over phosphoric anhydride at water pump vacuum. This material was a white powder.

The product gave a positive ninhydrin test for the free amino group. A modified Freudenberg acetyl analysis of this material indicated an acetyl content of 3.47 per cent which corresponded to about 0.4 acetyl groups per disaccharide unit. This analysis was conducted on undialyzed but purified material.

A carefully dried and weighed sample of this material was dialyzed against distilled water in a cellulose membrane of pore size such that particles of molecular weight greater than 8000 were retained (determined by Dr. W. W. Binkley of this laboratory). A total of 68.5 per cent of material was recovered after dialysis. This per cent recovery included mechanical loss. The final
product was dried under reduced pressure (water pump) over phosphoric anhydride at room temperature for 24 hours before weighing, as was the material before dialysis.

A sample of the above dialyzed material was submitted for elemental analysis. This analysis indicated that the material had an acetyl content of 0.6 acetyl groups per disaccharide unit, indicating that material lost during dialysis was more highly N-deacetylated. This was in agreement with findings regarding the low molecular weight of the minor N-deacetylation fraction discussed previously and substantiated the concept of the basic instability of N-deacetylated sodium chondroitin sulfate. Furthermore, the N-deacetylated sodium chondroitinsulfate, undialyzed, discussed here was sulfated by the pyridine chlorosulfonic acid method and the product of this reaction was dialyzed and submitted to both N-acetyl and elemental analysis. This material had an acetyl content corresponding to 0.6 acetyl groups per disaccharide unit. Therefore, the above evidence indicated that sodium chondroitin sulfate N-deacetylated by this method contained 60 per cent of its original acetyl. The optical rotation was $\left[\alpha\right]_{D}^{23} = -20.3^\circ$ (c 1.97, H$_2$O).

**Analysis:** Calculated for C$_{12}$H$_{16}$N$_{7}$O$_{10}$Na(SO$_2$Na$\cdot$2H$_2$O)$_{0.62}$ (C$_2$H$_3$O)$_{0.6}$; 33.57; H, 4.49; N, 2.97; Na, 7.89.
Found: C, 33.89; H, 4.00; N, 2.61; Na, 7.55 (a)

(b) Modification of Above Procedure:— Some slight variations were at times utilized in the above described deacetylation. However, these variations were not necessarily more effective than the one described. They are given here merely for completeness of presentation.

Firstly, it was found that the alkali concentration at the beginning of the reaction could be maintained at a higher value if the sodium chondroitinsulfate were dissolved in 10 ml. of 10 per cent sodium sulfite solution before addition to the alkali. This step eliminated the addition of 1 gram of aqueous sodium sulfate prior to the sample addition and thus the sodium hydroxide was less diluted with water. However, the dry nitrogen passing through the reaction solution probably removed enough water to maintain a high concentration value.

The second modification was in the separation of the N-deacetylated and precipitated sodium chondroitinsulfate from the alcoholic sodium hydroxide solution. A filter stick was attached to the bottom of the nitrogen inlet tube in the aeration flask (bubbling tower). The outlet tube was sealed and the liquid drawn off by a vacuum applied to the inlet tube. This was a simple

(a) This analysis was conducted by the Huffman Microanalytical Laboratories. The sample was dried at the B.P. ethanol in an oil pump vacuum over phosphoric anhydride to constant weight prior to analysis.
inverted filtration technique. Fresh methanol was admitted through the other tube and again withdrawn through the filter without the admission of air into the system. This technique was time consuming but did provide an air free system.

(c) **Qualitative Functional Tests.**

(1) **Ninhydrin:** About 1 mg. of partially \(\text{N}\)-deacetylated sodium chondroitinsulfate was dissolved in about 3 ml. of 50 per cent aqueous pyridine containing a very small amount of ninhydrin reagent (a few crystals were dissolved in the solution.) The resulting solution was boiled two minutes in a water bath. A violet color developed indicating free amino present.

(2) **Hexosamine:** To about 1 mg. of partially \(\text{N}\)-deacetylated sodium chondroitinsulfate in 1 ml. of distilled water was added 0.2 ml. of acetylacetone solution (1 ml. of acetylacetone in 25 ml. of 1 \(\text{N}\) sodium carbonate.) The solution was heated on a boiling water bath for fifteen minutes, cooled and 2.5 ml. of glacial acetic acid and 0.25 ml. of Ehrlich's reagent (0.8 g. of \(P\)-dimethylaminobenzaldehyde in 30 ml. of aldehyde free ethanol and 30 ml. of concentrated hydrochloric acid.) On heating a few minutes on a boiling water bath the solution developed a pink color indicating the presence of hexosamine in the material. This is the procedure
of Palmar, Smyth and Meyer (62).

(3) **Hexuronic Acid:**—The procedure of Dische (61) was used in this qualitative test. To about 1 mg. of N-deacetylated sodium chondroitinsulfate in 1 ml. of distilled water was added slowly and with cooling, 6 ml. of concentrated sulfuric acid. The solution was heated on a boiling water bath for twenty minutes, cooled and 9.2 ml. of a 0.1 per cent alcoholic carbazole solution was added with shaking. A pink color developed in a few minutes and intensified on standing. This indicated the presence of a uronic acid group.

(4) **Bound Sulfate:**—About 1 mg. of the material was dissolved in 2 ml. of distilled water in a pyrex test tube. About 1 ml. of 10 per cent barium chloride solution was added and the solution heated a few minutes on a boiling water bath. The solution remained clear indicating the presence of no free sulfate. The solution was acidified with a few drops of concentrated hydrochloric acid and heated a few minutes as before. A white precipitate of barium sulfate developed indicating the presence of acid-hydrolyzable sulfate.

(5) **Benedict's Reducing Test:**—Partially N-deacetylated sodium chondroitinsulfate was found to be slightly reducing to Benedict's solution when heated in a boiling water bath.

(a) Procedure:—An amount of 3.0048 grams of the sodium salt of chondroitinsulfate (purified as in III-A) was dissolved in 40 ml. of pure formamide (distilled). An amount of 50 ml. of distilled ethanolamine was added slowly with stirring. This homogeneous solution was poured into a small liquid-liquid extraction apparatus fitted with a condenser and outlet tube leading into alcoholic hydrogen chloride solution. To the extraction tube containing the formamide solution was added 3 drops of concentrated hydrochloric acid followed by pure ethyl ether until the tube was full (150 ml.). The side arm flask was filled one-half full of ethyl ether and the solution was extracted for 4 days. After a short induction period ammonia was continuously evolved from the outlet tube. After two days 20 ml. of ethanolamine was added to the lower layer and the extraction continued for a total of 4 days. The ether was replaced by dry toluene and the extraction continued. After 2 days 20 ml. more of ethanolamine was added to the lower layer. The toluene extraction was run a total of 4 days and the upper layer was removed. A tan oil had collected in the toluene side arm pot (B.P. 160°C.). The chondroitin solution was poured into 1 liter of ethanol and the precipitate was collected by filtration.
The white powder was dissolved in water (100 ml.) and precipitated by the addition of about 3 volumes of absolute ethanol. After allowing the precipitate to settle about 2 hours it was collected by centrifugation, dissolved in 100 ml. of distilled water and precipitated a second time by the addition of 3 volumes of absolute ethanol. The material was collected as before and again dissolved in 100 ml. of distilled water. This basic aqueous solution was dialyzed against distilled water for 2 days.

After dialysis the clear, light amber solution gave a very positive ninhydrin test as compared to previous samples of partially N-deacetylated chondroitinsulfate. It also gave a positive test for bound sulfate. The product was obtained by slowly adding three volumes of absolute ethanol to the aqueous solution, with stirring. The white flocculent precipitate was collected, washed with 85 per cent ethanol, 95 per cent ethanol, absolute ethanol and ethyl ether. The material was dried at room temperature in water pump vacuo over phosphoric anhydride, to yield a white powder. The yield of product was 2.5617 grams (85.5 per cent assuming formate salt of the amino group). This material gave a positive ninhydrin test and a positive test for bound sulfate.

This white powder was dissolved in 100 ml. of distilled water and the pH adjusted to 12.5 by addition of
dilute sodium hydroxide. The material was then precipitated by the addition of 3 volumes of absolute ethanol, with stirring. The product was collected by centrifugation. This product was redissolved in water and reprecipitated a total of 5 times. The five aqueous solutions in the process were basic. The pH dropped after each precipitation from 12.5 to 6.8 for the last obtained solution.

This sixth aqueous solution became cloudy on standing a few minutes and a white precipitate settled out. This precipitate was collected and was found to be soluble in slightly acid aqueous solutions but insoluble in aqueous solutions above pH 6.8. The remaining solution was mixed with three volumes of absolute ethanol and the white precipitate collected by centrifugation.

The above white powder was collected in two centrifuge tubes, washed with absolute ethanol and absolute ether and dried at room temperature in water pump vacuo over phosphoric anhydride.

An amount of 0.6643 g. of this material was dissolved in 50 ml. of distilled water and dialyzed for 24 hours against distilled water. The resulting solution was lyophilized to yield a pure white, fluffy material which gave an excellent ninhydrin test for a
free amino group as well as a positive test for bound sulfate. The material was dried at room temperature in water pump vacuo over phosphoric anhydride, yield 0.6448 grams (97.5 per cent).

A series of N-acetyl determinations were conducted on this material by the modified Freudenberg procedure (Sec. III-B-2). The acetyl content was found to be 2.12; 2.07 and 3.78 per cent respectively on three such determinations. The distillate from the latter determination was evaporated almost to dryness and heated with aqueous barium chloride solution. The solution became cloudy indicating that some sulfuric acid had distilled over during the analysis. The 20 per cent P-toluene-sulfonic acid solution of this N-desacetylated material in the boiling flask used in the analysis became cloudy upon heating a few minutes. This was probably the polymer precipitating from solution due to the loss of sulfate or carboxyl in the acid medium. This phenomenon was not encountered during acetyl determinations on other chondroitinsulfuric acid derivatives.

A sample of the final product was analyzed for carbon, hydrogen, nitrogen, sulfur and ash. In the calculations to follow the disaccharide repeating unit of disodium chondroitinsulfate was assumed to be that found in the analysis of this material \((\text{C}_{14}\text{H}_{19}\text{Na}_{11}\text{SO}_{3}\text{Na} \cdot 2\text{H}_2\text{O})_{0.745}\). The molecular weight of this repeating unit
was 504.187. The acetyl content, on the basis of one acetyl group per disaccharide unit, was 8.528 per cent acetyl. The two low acetyl determinations on the final N-deacetylated chondroitin sulfate were used to calculate the per cent deacetylation of the material. This calculation gave a value of 76.6 per cent deacetylation for the reaction. In this manner a theoretical repeating unit for the final product was derived and compared with the other analytical results. The optical rotation of this product was \( \alpha \sim \frac{\pi}{2}^\circ \) -22.1\(^\circ\) (c 0.972, H\(_2\)O).

\textbf{Analysis:} Calculated for (C\(_{12}\)H\(_{16}\)NO\(_{10}\)Na
(SO\(_3\)Na·H\(_2\)O)\(_{0.745}\) (C\(_2\)H\(_3\)O)\(_{0.234}\): C, 32.65; H, 4.22; N, 3.06;
0, 46.01\(^\circ\); S, 5.26; Na, 8.80; acetyl, 2.19. Found:
C, 32.72 (H); H, 3.79 (H); N, 2.62 (H); 0, 47.81;
S, 5.39 (H); Na, 8.48 (H); acetyl, 2.12 (b), 2.07 (b).

(H) Elemental analyses were conducted by the Huffman Microanalytical Laboratories, Wheatridge, Colorado. The material was dried in an oil pump vacuum over phosphoric anhydride to constant weight prior to analysis.
(b) Acetyl determinations were conducted in this laboratory after drying the samples at 78\(^\circ\)C. in an oil pump vacuum over phosphoric anhydride for 60 hours.
* Oxygen value was by difference in both cases.
(b) **Qualitative Functional Tests**

1. **Ninhydrin:** About 1 mg. of the above product was dissolved in 3 ml. of 50 per cent aqueous pyridine. A few crystals of ninhydrin were added and the resulting solution heated on a boiling water bath as previously described. After boiling for 2 minutes, an intense violet color developed, indicating strongly the presence of a free amino group. This test was comparable in intensity with an equivalent amount of D-glucosamine when tested under similar conditions. Also, the color given by this material was much more intense than that obtained from partially N-deacetylated sodium chondroitinsulfate, prepared by the concentrated alkali procedure, under similar conditions.

2. **Hexosamine:** When N-deacetylated sodium chondroitinsulfate prepared by this procedure was submitted to the previously described hexosamine test (Sec. III-B-5) a pink color developed, indicating the presence of hexosamine.

3. **Hexuronic Acid:** Submission of this N-deacetylated material to the previously described Dische uronic acid test produced a pink color indicating
the presence of uronic acid in the material.

(4) **Bound Sulfate**—A positive test for bound sulfate was obtained when this material was submitted to the previously described sulfate test (Sec. III-B-5).

(5) **Benedict's Reducing Test**—This N-deacetylated sodium chondroitinsulfate was found to be slightly reducing to Benedict's solution.

(6) **Solubility**—This material was less watersoluble than sodium chondroitinsulfate. Furthermore, in aqueous medium, solubility was a function of pH in that the material tended to precipitate from solution above pH 7.

(c) **Quantitative Analysis**

(1) **Acetyl Estimation**—This material was submitted to the p-toluenesulfonic acid acetyl determination as described in Sec. III-B-3. Partially N-deacetylated chondroitinsulfate, 0.1641 g., 0.1235 g. and 0.1478 g., required, respectively, 0.81 ml. of 0.1003 N sodium hydroxide, 1.47 ml. of 0.0400 N sodium hydroxide and 1.31 ml. of 0.1003 N sodium hydroxide to neutralize the distillate. These corresponded to 2.12, 2.07 and 3.78 per cent acetyl, respectively.
(2) **Elemental Analysis:** Elemental analysis on this material was conducted by the Huffman Microanalytical Laboratories, Wheatridge, Colorado.
C. Sulfation of N-Deacetylated Chondroitin Sulfuric Acid and Properties of the Product

1. Employing Pyridine and Chlorosulfonic Acid.

(a) Procedure:—An amount of 3.0 grams of finely ground chondroitin sulfate, previously N-deacetylated by the strong alkali method (Sec. III-B-5) but not dialyzed, was dissolved in 50 ml. of distilled water. This material was then precipitated by the addition of 3 volumes of absolute methanol. The white flocculent precipitate was allowed to settle and most of the clear solution removed by decantation. An equal volume of pyridine (distilled) was added carefully to the solution containing the precipitate. This was done with stirring. It was found that the direct addition of pyridine to the clear aqueous solution caused the chondroitin sulfate to precipitate as a sirup. The precipitate was collected by centrifugation. This gelatinous precipitate was washed 5 times with dry pyridine. Each washing was conducted by filling the tube containing the precipitate with dry pyridine followed by stirring to suspend the material. The tube was then centrifuged and the pyridine decanted off. After 5 washings the tube was again filled with dry pyridine, the material suspended, and placed in a desiccator at room temperature over phosphoric anhydride. The suspended chondroitin sulfate was now a tan gel.
A one hundred milliliter 3-necked flask was fitted with a reflux condenser, on which was placed a calcium chloride drying tube, a mechanical stirrer and a dropping funnel. An amount of 45 ml. of distilled and dry pyridine was added to this container and cooled in an ice bath. To this cooled solution was added 6 ml. of distilled chlorosulfonic acid with stirring. The white precipitate that formed was dissolved by heating the solution to about 40°C. The chondroitinsulfate, suspended in about 20 ml. of dry pyridine, was poured into the stirred solution. The reaction temperature was maintained at 80-90°C for 1 hour and the solution allowed to cool. A tan syrup collected in the bottom of the flask. Most of the pyridine solution was decanted off and the remaining solution diluted with cold dilute sodium hydroxide solution until the syrup dissolved. This solution was cooled in an ice bath without removing from the flask and carefully neutralized with 0.1 N sodium hydroxide. The resulting tan solution was immediately poured into 1 liter of absolute ethanol and the flocculent precipitate allowed to settle. This material was collected by centrifugation and redissolved in 100 ml. of distilled water followed by precipitation with methanol. At least three such precipitations were conducted and the final material dissolved in 100 ml. of distilled water and dialyzed against distilled water for two days, in a membrane of pore size specified on page 70.
The final solution was filtered through an ultrafine sintered glass funnel and the product precipitated by the addition of 3 volumes of methanol. Precipitation began after the addition of one volume of methanol. This precipitate was collected and washed with 95 per cent ethanol, absolute ethanol and finally, dry ether. It was dried at room temperature in water pump vacuo over phosphoric anhydride. The yield was 2.6 grams. The acetyl content was found to be 3.58 per cent. This value corresponded to 0.59 acetyl groups per disaccharide repeating unit of molecular weight 746.3. The final product was a light tan powder; $\delta = 27\text{Hg} -19.9^\circ$ (c 2.0, H$_2$O, saccharimeter). Elemental analysis on this material was conducted by the Huffman Microanalytical Laboratories, Wheatridge, Colorado. The material was dried in oil pump vacuo at 25°C. to constant weight over phosphoric anhydride before analysis. Acetyl determinations were conducted in this laboratory.

**Analysis:** Calculated for C$_{12}$H$_{13}$O$_{36}$N$_{10}$Na$_3$O$_{5}$ (SO$_3$Na)$_{3.55}$ (C$_2$H$_3$O)$_{0.59}$: C, 21.20; H, 2.11; H, 1.88, Na, 14.01; S, 15.25, acetyl, 3.40. Found: C, 21.54 (H); H, 2.50 (H); H, 1.96 (H) Na, 13.23 (H); S, 15.96 (H), 16.3; acetyl 3.58.

(H) Huffman Microanalytical Laboratories, Wheatridge, Colorado.
(b) **Qualitative Functional Tests on Sulfated Sodium Chondroitin Sulfate.**

(1) **Sulfate:** Approximately 1 mg. of sulfated chondroitin sulfate was dissolved in 2 ml. of distilled water in a pyrex test tube. A few drops of aqueous barium chloride solution were added and the solution heated on a boiling water bath for 20 minutes. No precipitate of barium sulfate appeared. The solution was acidified with concentrated hydrochloric acid and the heating continued. A voluminous precipitate of barium sulfate appeared indicating that only bound sulfate was present in the material.

(2) **Benedict's Reducing Test:** About 1 mg. of sulfated sodium chondroitin sulfate was dissolved in 2 ml. of distilled water and 3 ml. of Benedict's solution was added. On heating this solution to boiling for several minutes a slight copper precipitation occurred. This indicated that the material was slightly reducing.

(3) **Ninhydrin Free Amino Group Test:** About 1 mg. of sulfated sodium chondroitin sulfate was dissolved in 2 ml. of distilled water in a pyrex test tube. About 3 ml. of 50 per cent aqueous pyridine was added and the tube shaken. About 1 mg. of triketohydrindine hydrate was added to the solution and dissolved by shaking. On heating to boiling for two minutes the solution did not become colored indicating that no free amine was present. Extensive boiling eventually produced a purple color.
(4) **Hexuronic Acid**:- The procedure of Dische (61) was used in this qualitative test. To about 1 mg. of sulfated sodium chondroitinsulfate in 1 ml. of distilled water was added slowly, and with cooling, a 6 ml. portion of concentrated sulfuric acid. The solution was heated on a boiling water bath for 20 minutes, cooled and 9.2 ml. of a 0.1 per cent alcoholic carbazole solution was added with shaking. A pink color developed in a few minutes and intensified on standing. This indicated the presence of a uronic acid group.

(5) **Hexosamine**:- The procedure of Palmer, Smyth and Meyer (62) was used. To about 1 mg. of sulfated sodium chondroitinsulfate in 1 ml. of distilled water was added 0.2 ml. of acetylacetone solution (1 ml. of acetylacetone in 25 ml. of 1 N, sodium carbonate). The solution was heated on a boiling water bath for 15 minutes, cooled and 2.5 ml. of glacial acetic acid and 0.25 ml. of Ehrlich's reagent (0.8 g. of P-dimethyl-aminobenzaldehyde in 30 ml. of aldehyde-free ethanol and 30 ml. of concentrated hydrochloric acid,) were added. On heating a few minutes on a boiling water bath the solution developed a pink color indicating the presence of hexosamine in the material.

(6) **Acid Reaction**:- An aqueous solution of sulfated chondroitinsulfate was neutral to litmus.
(c) **Quantitative Analysis of Sulfated Sodium Chondroitinsulfate.**

(1) **N-Acetyl Estimation:** The analysis employed was a modification of the Freudenberg (20) procedure as employed by Wolfrom, Weisblat, Karabinos, McNeeley and McLean (18) and described in Sec. III-B-2. An 0.1513 gram sample of the sulfated sodium chondroitinsulfate discussed here yielded on distillation enough acetic acid to neutralize 6.71 ml. of 0.01875 N. NaOH. This corresponded to an acetyl content of 3.58 per cent or 0.6 acetyl groups per disaccharide unit.

(2) **Sulfate Estimation:** Semi-micro sulfate determinations were conducted, in this laboratory by the well known Parr bomb method. This determination indicated a sulfur content of 16.3 per cent. A micro sulfur determination by Dr. E. W. D. Huffman indicated a value of 15.96 per cent sulfur which corresponded to about 3.55 sulfate groups per disaccharide unit.

(3) **Anticoagulant Activity of N-Deacetylated and Sulfated Sodium Chondroitinsulfate:** The bioassay used in this laboratory was a modification of the procedure of Kuizenga, Nelson and Cartland (113, 114, 115). Frozen

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(114) O. F. Swoap and M. H. Kuizenga, J. Am. Pharm. Assoc., 38, 563 (1949)

citrated sheep plasma used in this analysis was obtained from the Wilson Laboratories, Chicago, Illinois.

The method is concerned with the comparison of the minimum amounts of standard heparin and of sulfated N-deacetylated sodium chondroitin sulfate contained in a volume of 0.4 ml. which will keep 1 ml. of recalcified sheep plasma more than 50 per cent fluid for 1 hour at room temperature. The amount of calcium chloride solution (20 mg./l ml.) required for recalcification of the thawed and filtered plasma is defined as that amount added to 1 ml. of plasma which will bring about complete coagulation of the plasma in the shortest possible time. In each case the total volume is made up to 1.4 ml. by the addition of the required amount of 0.9 per cent sodium chloride solution. Usually, about 0.1 ml. of calcium chloride solution is required per ml. of plasma. This is determined first for a given plasma sample.

Standard heparin solution (50 U/l ml. and sulfated N-deacetylated sodium chondroitin sulfate solution were made up in 0.9 per cent aqueous sodium chloride. The above concentration for the heparin solution was in agreement with that stated as the Provisional International Heparin Reference.

A series of well cleaned 11 x 100 mm. test tubes fitted with waxed cork stoppers were assembled and to each was added 1 ml. of plasma followed by varying
amounts of accurately measured heparin solution (usually 0.10 - 0.30 ml.) and followed by sufficient 0.9 per cent sodium chloride solution to give a total volume of 1.4 ml. followed by the above determined amount of calcium chloride solution. Each tube was immediately stoppered and inverted 3 times. At the end of one hour the series of tubes was examined for coagulation and that tube which was more than 50 per cent fluid was selected as the standard concentration. This concentration of heparin was preferentially near the center of the series of ascending or descending concentrations. The above process was then repeated with the quantitatively prepared sulfated sodium chondroitinsulfate solution.

Calculation of the relative activity of the sulfated N-deacetylated chondroitinsulfate was based on the fact that the concentration of this material in the test tube selected as most nearly 50 per cent clotted after one hour was known. Standard heparin has an activity of 110 International units per milligram and the concentration of heparin in that tube in the former series which was most nearly 50 per cent fluid after one hour was known. Therefore, the activity of the sulfated N-deacetylated sodium chondroitinsulfate was:

\[
\text{anticoagulant activity} = 110 \text{ I.U.} \times \frac{y_{\text{of sulfated N-deacetylated sodium chondroitinsulfate in the selected tube}}}{y_{\text{of heparin in the selected tube}}}
\]
A series of seven preparations of sulfated N-deacetylated sodium chondroitinsulfate prepared by this sulfation procedure had activities of 53, 55, 57, 53, 45, 52 and 39 International Units per mg. when evaluated by this procedure. A sample of sodium chondroitinsulfate was sulfated by the procedure and found to have an activity of less than 10 International Units per mg. That particular preparation of sulfated N-deacetylated sodium chondroitinsulfate for which analytical data have been given in this discussion had an activity of 57 International Units per mg.

(4) Toxicity of N-Deacetylated and Sulfated Sodium Chondroitinsulfate:—The acute toxicity of N-deacetylated sulfated sodium chondroitinsulfate were conducted by Dr. H. Leo Dickison, Director of Pharmacologic Research, Bristol Laboratories, Inc., Syracuse, N. Y. These toxicities were done in mice and 3 samples of the above material were submitted for study.

In every instance it was noted that the mice hemorrhaged from the nose and had bloody urines. It was stated that the bloody urines probably indicated a certain amount of hemolysis of the red cells. This is a common finding with high molecular weight polysaccharides. The toxicity values for this series of preparations was stated to be of the same general range as that of heparin.
Using the de Beer graphic method, the following results were calculated.

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Solution</th>
<th>I.V. mouse ( \text{LD}_{50} ) (mg. per kg.) ( ^a )</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.G.S. I</td>
<td>2% aq.</td>
<td>579 ± 66.63</td>
<td>13.8</td>
</tr>
<tr>
<td>C.G.S. II</td>
<td>2% aq.</td>
<td>726.1 ± 27.59</td>
<td>32</td>
</tr>
<tr>
<td>C.G.S. III</td>
<td>2% aq.</td>
<td>787 ± 15.74</td>
<td>68</td>
</tr>
</tbody>
</table>

\( \text{LD}_{50} \) = lethal dose (in mg. of substance, injected intravenously, per kg. of body weight of animal) required to kill 50% of the animals (white mice).

Clinical Anticoagulant Analysis of Sulfated N-Deacetylated Sodium Chondroitinsulfate: Clinical anticoagulant studies of this material were conducted by Dr. H. Leo Dickison, Director of Pharmacologic Research, Bristol Laboratories, Inc., Syracuse, N. Y. The first series of studies consisted of the comparison of the anticoagulant effect of this material as compared with heparin as supplied by Hynson, Westcott and Dunning, Inc. Comparison was made using fresh blood.

Two series of erlenmeyer flasks were set up and 0.5 ml. of a test solution was added to a flask. The test solutions had concentrations of 0.5 mg., 1.0 mg., 1.5 mg. and 2.0 mg. per milliliter of heparin in one series and sulfated N-deacetylated sodium chondroitinsulfate in the other series. An amount of 5.0 ml. of freshly drawn
rabbit's blood was then added to each flask. The flasks were gently rotated at intervals and the time of coagulation of each flask noted. The experiment was conducted three times.

It was found that 1 mg. of the sulfated N-deacetylated chondroitinsulfate prevented clotting from 18-24 hours while clotting occurred in less than 16 hours in all lesser concentrations of this material. On the other hand, 1 mg. of heparin prevented clotting for a period of almost 72 hours and lesser concentrations prevented clotting for a longer period than did the above lesser concentrations of sulfated material.

A second series of studies was conducted by the above cited laboratory on three preparations of sulfated N-deacetylated sodium chondroitinsulfate. In this study of plasma coagulation time of these preparations as compared with heparin, the method employed was that of Haley and Stolarsky (116). The data obtained by this method using rabbit blood and dog blood are given in the following Table VII.

Table VII

Coagulation Time of Sulfated N-Deacetylated Sodium Chondroitinsulfate

A. Rabbit Blood

Experiment I.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$10^{-5}$ dilution</th>
<th>$10^{-6}$ dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>160-200</td>
<td>50-60</td>
</tr>
<tr>
<td>C.G.S.-I a</td>
<td>100</td>
<td>62</td>
</tr>
<tr>
<td>C.G.S.-II</td>
<td>98-109</td>
<td>63</td>
</tr>
<tr>
<td>C.G.S.-III</td>
<td>93-105</td>
<td>61-65</td>
</tr>
</tbody>
</table>

Control plasma clotted in 50-58 seconds.

Experiment II.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$10^{-5}$ dilution</th>
<th>$10^{-6}$ dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>145-148</td>
<td>50-60</td>
</tr>
<tr>
<td>C.G.S.-I</td>
<td>85-113</td>
<td>47-55</td>
</tr>
<tr>
<td>C.G.S.-II</td>
<td>85-93</td>
<td>55-62</td>
</tr>
<tr>
<td>C.G.S.-III</td>
<td>90-92</td>
<td>60</td>
</tr>
</tbody>
</table>

Control plasma clotted in 40-57 seconds.

B. Dog Blood

Experiment III.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$10^{-5}$ dilution</th>
<th>$10^{-6}$ dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>140-155</td>
<td>65</td>
</tr>
<tr>
<td>C.G.S.-I</td>
<td>70-83</td>
<td>50-60</td>
</tr>
<tr>
<td>C.G.S.-II</td>
<td>74-83</td>
<td>57-64</td>
</tr>
<tr>
<td>C.G.S.-III</td>
<td>80-93</td>
<td>50-58</td>
</tr>
</tbody>
</table>

Control plasma clotted in 45-50 seconds.

a The symbols C.G.S.I,II,III refer to the three preparations of sulfated N-deacetylated sodium chondroitinsulfate prepared by this worker. Sample C.G.S.-II is that preparation for which extensive analytical data have been given in this report.
2. Homogeneous Sulfation in Formamide*

(a) Procedure:—An amount of 1.6347 g. of dry N-de-acetylated chondroitinsulfate, prepared by the strong alkali procedure (III-B-5) was dissolved in 30 ml. of dry distilled formamide. Solution was obtained by stirring the mixture in a three-necked flask fitted with a mechanical stirrer, dropping funnel and a water condenser fitted with a moisture trap. The stirred solution was then cooled in an ice bath and 5.74 ml. of dry pyridine was slowly added. When the solution clarified, 2.3 ml. of distilled chlorosulfonic acid was added dropwise. At this time the solution was allowed to warm to room temperature. After 10 hours an additional 4.6 ml. of dry chlorosulfonic acid was added and the solution stirred overnight. An additional 2.0 ml. of dry chlorosulfonic acid was added when the reaction had gone 21 hours. At the end of 24 hours, the clear amber solution was neutralized with N sodium methoxide to a phenolphthalein end point (internal). A flocculent white precipitate formed and this was collected and washed with ethanol. This white powder was precipitated twice from distilled water by the addition of absolute ethanol and was then dissolved in 50 ml. of distilled water.

The aqueous solution was dialyzed for two days against distilled water in a membrane which maintained material of molecular weight greater than 8000. The final product

* This experiment was conducted in a hood due to the possible danger of hydrogen cyanide gas.
was precipitated from the water solution by the addition of about 3 volumes of absolute ethanol/methanol (1/1).

It was collected by centrifugation and washed well with ethyl alcohol, methyl alcohol and ethyl ether. This very light powder was dried at room temperature in water pump vacuo over phosphoric anhydride. A sample of partially N-deacetylated chondroitinsulfate, from the same lot used in this experiment, was dialyzed for 2 days and it was found that 40 per cent of the material passed through the membrane. The yield on this sulfation experiment was 1.4035 grams (95.5 per cent of theory): \( \Delta_{\text{240}} -18.63^\circ \) (c 1.56, \( H_2O \)).

Analysis(a): Calculated for \( C_{12}H_{15}NO_{10}(SO_4Na)_3.4 \) \( (C_2H_3O)_{0.6} \): C, 22.34; H, 2.39; S, 15.37; Na, 11.02; Acetyl, 3.65. Found: C, 21.97 (d), 22.30 (d); H, 2.97 (d), 2.85 (d); S, 14.87 (H), 15.06 (H); Na, 11.92 (H), 11.12 (d), 11.22 (d); acetyl 3.73, 3.82 (c).

(b) Qualitative Tests (I) Sulfate: The test for bound sulfate was conducted as described in the previous section. This material gave a very positive test for bound sulfate. The material did not contain any inorganic

(a) Material was dried as specified in Sec. III-A for respective analysts.
(d) Departmental Analyst, W. H. Deebel.
(H) Huffman Microanalytical Laboratories, Wheatridge, Colorado.
(c) Acetyl content found for sulfated N-deacetylated sodium chondroitinsulfate prepared from the same batch of N-deacetylated material sulfated by the pyridine-chloro-sulfuric acid procedure.
sulfate.

(2) **Benedict's Reducing Test:** This preparation of sulfated chondroitinsulfate was slightly reducing to Benedict's solution.

(3) **Hydrazin Amino Group Test:** This sulfated chondroitinsulfate was tested for free amino groups as previously described. The material gave a negative test on heating the required time but gave a positive test on continued boiling for a few minutes. This positive test appeared more quickly than in the previous preparation. This would indicate that some of the instability characteristic of heparin was present in this material.

(4) **Hexuronic Acid:** The procedure previously described was used in this qualitative test. A pink color developed when this material was treated with alcoholic carbazole. This color darkened somewhat on standing for a few hours, indicating uronic acid.

(5) **Hexosamine:** This sulfated and N-deacetylated sodium chondroitinsulfate gave a pink color when submitted to the previously described test of Palmer, Smythe and Meyer. This confirmed the presence of hexosamine in the polymer.

(6) **Acid Reaction:** Unlike the first preparation, this sulfated N-deacetylated sodium chondroitinsulfate, when dissolved in distilled water, was possibly slightly acid to litmus.
(c) Quantitative Analysis of N-Deacetylated and Sulfated Sodium Chondoritinsulfate.

(1) N-Acetyl Estimation:—Due to the limited amount of material on hand this sample was not submitted to the Freudenberg acetyl analysis procedure as described in SEC. III-B-2. However, a run of N-deacetylated and sulfated sodium chondroitinsulfate prepared by the heterogeneous sulfation procedure was analyzed for N-acetyl. This material had an acetyl content of 3.73 per cent and 3.82 per cent, respectively, on duplicate determinations on 0.1713 g. and 0.1432 g. samples whose distillates required 7.31 and 8.36 ml. of 0.1000 N sodium hydroxide to neutralize. Both sulfated derivatives were prepared from the same batch of N-deacetylated chondroitinsulfate and there is no evidence on hand that the chlorosulfonic acid sulfation procedure causes any appreciable N-deacetylation of the material. Other analytical data on the material indicated an acetyl content of about 3.65 per cent or 0.6 groups per disaccharide unit.

(2) Anticoagulant Activity:—This material was submitted to the sheep plasma bioassay as discussed in the section concerned with the previous sulfation procedure. The assay was run on two different shipments of sheep plasma and the material was found to have an anticoagulant activity of 41.4 and 23 International Units per
milligram for the two determinations. The second determination was made using plasma which was older than the former run and time did not permit complete evaluation.

D. The Acetylation of Sodium Chondroitinsulfate

1. Procedure: An amount of 3.83 g. of sodium chondroitinsulfate, purified as described in Sec. III-A-1), was finely pulverized and dried at room temperature in water pump vacuo over phosphoric anhydride, for 2 days. This dry powder was dissolved in 24 ml. of dry, freshly distilled formamide by stirring overnight in a three-necked flask fitted with a dropping funnel, mechanical stirrer and a reflux condensor fitted with a moisture trap. An amount of 24 ml. of dry, freshly distilled pyridine was added with stirring followed by 10 ml. of acetic anhydride. The reaction mixture was allowed to come to room temperature. This solution was stirred for 12 hours. An amount of 13 ml. of acetic anhydride was added slowly with stirring. The resulting solution was stirred for a total of 24 hours. No appreciable darkening of the solution resulted (medium red color).

At the end of 24 hours this solution was poured into an amount of 500 ml. of absolute ethanol, cooled in an ice bath, with stirring. An amount of 400 ml. of absolute ethanol was then added to yield a white, flocculent precipitate. This precipitate was collected by filtration
and washed well with ethanol. The washed precipitate was dissolved in 100 ml. of distilled water. This solution was poured into 500 ml. of absolute ethanol. Precipitation occurred on the addition of a few ml. of saturated aqueous sodium chloride solution (3-5 ml.). This precipitate was collected and the process was repeated twice.

The final product was dissolved in 100 ml. of water and dialyzed for 2 days against distilled water to remove any sodium chloride present. It was noted that the product was more soluble in water-ethanol than sodium chloride, unlike sodium chondroitinsulfate. After dialysis the product was collected by freeze-drying to remove the water. The material was dried, at room temperature over phosphoric anhydride and at water pump vacuum, for 2 days to yield a very fluffy, white material. The yield was 3.62 grams. This yield was over 89.5 percent based on the calculated yield of the tetraacetate of sodium chondroitinsulfate. An infrared spectrogram of this material produced excellent absorption peaks for O-acetyl and N-acetyl groups. The optical rotation for the first dialyzed material was $\eta_{271}D -27.85^\circ$ (c 1.63, H$_2$O).

The analysis of this material is given in Table VIII. Two preparations are given for comparison. The first analysis is that of a nondialyzed preparation while the
Table VIII
Analysis of Acetylated Chondroitinsulfate Disodium Salt

<table>
<thead>
<tr>
<th>Found</th>
<th>Required</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tetra-(a) Per-acetate</td>
</tr>
<tr>
<td>I %</td>
<td>II %</td>
</tr>
<tr>
<td>C</td>
<td>38.41, 37.83(^D)</td>
</tr>
<tr>
<td>H</td>
<td>4.91, 4.53(^D)</td>
</tr>
<tr>
<td>N</td>
<td>2.44, 2.34(^D)</td>
</tr>
<tr>
<td>Na</td>
<td>5.94, 6.14(^D)</td>
</tr>
<tr>
<td>Tot. acetyl</td>
<td>26.90, 27.04</td>
</tr>
</tbody>
</table>

(a) \(C_{12}H_{13}NO_\text{Na}(C\text{H}_3O)(SO_3\text{Na} \cdot 2\text{H}_2\text{O})\cdot 0.745\text{(H)} \cdot 0.255\) repeating unit; mol. wt., 630.26.
(b) \(C_{12}H_{13}NO_\text{Na}(C\text{H}_3O)\cdot 4.255(SO_3\text{Na} \cdot 2\text{H}_2\text{O})\cdot 0.745\) repeating unit; mol. wt., 641.24.
(c) Acetyl determinations were conducted in this laboratory.
(D) Departmental Analyst, W. H. Deebele.
(H) Huffman Microanalytical Laboratories, Wheatridge, Colorado.
second is that of a dialyzed preparation. The low ash content in the latter may be due to loss of sodium ion during dialysis. The basic calculated repeating unit is taken directly from the analysis of the starting sodium chondroitinsulfate. Samples for elemental analysis were dried for the respective analysts as described in Sec. III-A.

2. **Qualitative Tests**

(a) **Sulfate:** Approximately one milligram of acetylated chondroitinsulfate was dissolved in 2 ml. of distilled water in a small pyrex test tube. A few drops of aqueous barium chloride solution were added. After heating for 20 minutes on a boiling water bath, no barium sulfate precipitate had formed, showing no free sulfate to be present. About 3 drops of concentrated hydrochloric acid were added and the heating was continued. In about 15 minutes a white precipitate of barium sulfate formed, showing that acid-hydrolyzable sulfate was present. This white precipitate was not soluble in concentrated hydrochloric acid.

(b) **Benedict's Reducing Test:** About 1 milligram of acetylated chondroitinsulfate was dissolved in about 2 milliliters of distilled water and 3 milliliters of Benedict's solution was added. On heating this solution to boiling for one minute, no copper was precipitated. On standing for 1 hour, no copper was precipitated.
Therefore, acetylated (but deacetylated in the test) chondroitinsulfate is non-reducing.

(c) Ninhydrin Amino Test:—About one milligram of acetylated chondroitinsulfate was dissolved in 2 ml. of distilled water in a small Pyrex test tube. About 3 ml. of 50 per cent aqueous pyridine and about 1 milligram of triketohydrindine hydrate was added. The solution was shaken until the ninhydrin had dissolved and then heated on a boiling water bath for 3 minutes. The solution remained colorless, indicating that no free amino groups were present.

(d) The Sodium Iodide-Barium Iodide Test:—A few milligrams of acetylated chondroitinsulfate were dissolved in about 5 ml. of 10 per cent aqueous barium iodide solution. A few milligrams of sodium iodide were added and the solution was set aside in a stoppered test tube for 2 days. At the end of this time, a white precipitate of barium sulfate had formed. This white precipitate was not soluble in concentrated hydrochloric acid.

3. Acetyl Analysis

The acetyl analysis used in this work was a modification of the original Freudenberg procedure (20) as described in Sec. III-B-2. Two samples of acetylated chondroitinsulfate (0.1125 g. and 0.1284 g.) produced acetic acid which consumed 7.31 and 8.32 ml. respectively of 0.1000 N sodium hydroxide. These corresponded
to 27.8 and 28.05 per cent acetyl respectively.

E. The Sulfation of Ethanolamine

1. The Pyridine-Sulfur Trioxide Method

(a) Procedure:—An amount of 7.5 ml. of commercial stabilized sulfur trioxide* was added slowly and with

* "Sulfan B", General Chemical Div., Allied Chemical and Dye Corp., New York, N.Y.

stirring to 30 ml. of distilled pyridine in a three-necked flask fitted with a dropping funnel, mechanical stirrer and a moisture sealed reflux condensor. This addition was carried out over a period of 1 hour and a voluminous precipitate formed. An additional 10 ml. of pyridine was added to dilute the very thick solution. To the mixture, cooled in an ice bath, was added dropwise and with stirring, an amount of 5 ml. of freshly distilled ethanolamine over a period of 4 hours. The original solid mass slowly dissolved and a new one slowly formed in the stirred mixture. The mixture was then heated to 60°C. for about 30 minutes, with stirring, and let stand at room temperature for an additional 24 hours.

The pyridine upper layer was decanted from the semi-solid residue in the bottom of the flask. Approximately the calculated amount of N sodium methoxide solution was poured directly into the flask from a dropping funnel.
It was hoped that the very acid product could be neutralized with base without being in contact with moisture. Two drops of alcoholic phenolphthalein indicator were added at the beginning of the neutralization and the solution was stirred vigorously with outside cooling to prevent overheating. The addition of sodium methoxide was controlled so as not to cause the solvent to boil. A voluminous white precipitate formed and this was collected by filtration. A small additional quantity of product was obtained by evaporation of the methanol. The two products were combined, washed well with ethyl ether and dried at room temperature over phosphoric anhydride at water pump vacuum. This crude product gave a very weak ninhydrin test for free amine. Total yield of the white amorphous powder was 19.1 grams.

Several grams of the crude product were consumed in attempts to prepare a pure crystalline product. The material was very soluble in water, slightly soluble in absolute methanol and was insoluble in absolute ethyl ether. Very slow or rapid evaporation of dilute methanol solutions of this material at various temperatures did not result in a crystalline product. Very careful precipitation by the slow addition of absolute ethyl ether to a dilute methanol solution was not successful. The remaining crude product was dissolved in methanol and precipitated by the addition of ethyl ether to give a
white powder. This powder gave a negative ninhydrin test.

After much experimentation it was found that this disodium salt would crystallize as a hydrate. About one gram of the material was dissolved in 20 ml. of distilled water and 9 volumes of c.p. methyl alcohol were added. The cloudy solution was centrifuged and c.p. ethyl ether was added with stirring until the clear solution became cloudy. This solution was stored in a stoppered flask at 0°C for one week. At the end of this time a semicrystalline mass had formed in the bottom of the flask. Part of this material was reserved for seeding purposes and the remainder was dissolved in 5 ml. of distilled water and 9 volumes of methyl alcohol were added with swirling of the flask. Ethyl ether was added dropwise with stirring until the solution became very slightly cloudy. The seeding crystals were added and the flask was scratched very vigorously with a glass stirring rod. The addition of an excess of ethyl ether, or even a too rapid addition, at this point invariably led to the precipitation of the product as a white amorphous powder. Under the previously stated conditions, ethanolamine disulfate almost immediately began to crystallize as very delicate needles which soon became platelets. The solution was sealed and let stand at room temperature for 1 day. Subsequent addition of
small amounts of ethyl ether caused additional material to crystallize. The crystalline material was collected by careful filtration and washed with ethyl ether. It was then recrystallized twice as described above and dried in the presence of atmospheric moisture. It was found that the material could be dried in a desiccator over calcium chloride without loss of water of hydration; yield 0.9 g.

This final purified product had a sharp melting point at 220-221°C. (uncorr.). However, rapid heating caused water of hydration to be evolved at about 80°C. The resulting white powder then remelted at about 222°C. An x-ray powder diagram was prepared of this material and is shown in Figure 1. Several distinct lines were produced and these are recorded in Table IX. A sample was stored in contact with atmospheric moisture for 3 days and a complete elemental analysis was conducted. Water of hydration was found by by drying the hydrate at 110°C. in vacuo to constant weight. The other determinations were conducted on this dry basis.

Analysis (a): Calculated for C₂H₅N⁺Na₂S₂O₇: C, 9.06; H, 1.90; N, 5.28; S, 24.18; Na, 17.34. Found: C, 9.11; H, 1.69; N, 4.97; S, 23.80, 23.82; Na, 17.64 (as Na₂SO₄).

Calculated for C₂H₅N⁺Na₂S₂O₇·3H₂O: H₂O, 16.93

(a) Analysis was conducted by Huffman Microanalytical Laboratories, Wheatridge, Colorado.
Figure 1. X-Ray Diffraction Pattern of the Trihydrate of 2-Sulfaminoethylsulfuric Acid, Disodium Salt.
## Table IX

X-Ray Powder Diffraction Pattern of the Trihydrate of the Disodium Salt of Ethanolamine Disulfate

<table>
<thead>
<tr>
<th>2θ</th>
<th>d, Å</th>
<th>Intensity (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.48</td>
<td>11.819</td>
<td>Strong</td>
</tr>
<tr>
<td>9.70</td>
<td>9.1178</td>
<td>Very strong</td>
</tr>
<tr>
<td>13.93</td>
<td>6.3572</td>
<td>Medium</td>
</tr>
<tr>
<td>16.30</td>
<td>5.4378</td>
<td>Strong</td>
</tr>
<tr>
<td>19.73</td>
<td>4.5025</td>
<td>Strong</td>
</tr>
<tr>
<td>20.48</td>
<td>4.3364</td>
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</tr>
<tr>
<td>21.55</td>
<td>4.1234</td>
<td>Weak</td>
</tr>
<tr>
<td>23.00</td>
<td>3.8667</td>
<td>Very strong</td>
</tr>
<tr>
<td>24.05</td>
<td>3.6992</td>
<td>Weak</td>
</tr>
<tr>
<td>25.05</td>
<td>3.5547</td>
<td>Strong</td>
</tr>
<tr>
<td>27.80</td>
<td>3.2090</td>
<td>Medium</td>
</tr>
<tr>
<td>28.83</td>
<td>3.0966</td>
<td>Weak</td>
</tr>
<tr>
<td>29.58</td>
<td>3.0298</td>
<td>Medium</td>
</tr>
<tr>
<td>30.43</td>
<td>2.9374</td>
<td>Very diffuse</td>
</tr>
<tr>
<td>32.38</td>
<td>2.7648</td>
<td>Weak</td>
</tr>
<tr>
<td>33.40</td>
<td>2.6827</td>
<td>Weak</td>
</tr>
<tr>
<td>35.53</td>
<td>2.5266</td>
<td>Strong</td>
</tr>
</tbody>
</table>

(a) Estimated visually.
(b) Qualitative Functional Tests

(1) Ninhydrin:- About 1 mg. of the disodium salt of ethanolamine disulfate was dissolved in 1 ml. of distilled water in a small test tube. A few crystals of trilcetohydindene hydrate were added followed by 2 ml. of 50 per cent aqueous pyridine. The tube was shaken until the reagent had dissolved and the solution was then heated for 2 minutes in a boiling water bath. No purple color, indicative of a free amino function, developed. Prolonged heating produced a weak positive test. Control samples of ethanolamine and "β-aminomethylsulfate" (*) were tested simultaneously. Both of the latter compounds gave very positive tests.

(2) Sulfate:- About 1 mg. of the sodium salt of ethanolamine disulfate was dissolved in a 5 ml. quantity of distilled water and a few drops of aqueous 10 per cent barium chloride were added. The solution remained almost clear. However, the addition of a few drops of concentrated hydrochloric acid followed by heating caused the solution to become much more turbid. This was expected because the barium salt of the compound was not too water soluble. And thus acid hydrolyzable sulfate was present.
(c) Quantitative Analysis

Sulfur: Due to the small amount of purified material obtained, micro sulfur determinations were run by Dr. E. W. D. Huffman. These determinations showed that two equivalents of sulfur were present per ethanolamine group.

X-Ray Powder Diffraction Pattern: An x-ray powder diffraction pattern was prepared of the hydrate of the disodium salt. Film exposure time was two hours. Filtered CuKα radiation, effectively A°, was employed with a 114.59 mm. camera. The resulting photograph is presented in Figure 1 and the data are in Table IX. Measurements of the line radii were recorded in millimeters and these were converted to d spacings by means of the appropriate table (117).


2. Attempted Sulfation of Ethanolamine by the Pyridine/Chlorosulfonic Acid Method

(a) Procedure: An amount of 10 ml. of distilled chlorosulfonic acid was added with stirring and cooling to 40 ml. of anhydrous pyridine in a three-necked flask fitted with a mechanical stirrer, a dropping funnel and
a moisture sealed reflux condenser. The solution was heated to 60°C to dissolve the white precipitate which had formed and 3 grams of distilled ethanolamine were added dropwise. The resulting solution was maintained at 50°C for 3 hours and cooled to room temperature.

An amount of 20 ml. of the above sulfation mixture was neutralized to pH 7 with 0.2 N sodium methoxide. A white gelatinous precipitate formed. The precipitate was collected and extracted with 200 ml. of methanol. This methanol extract was reduced in volume to 30 ml. when a white precipitate formed. This precipitate was collected and the supernatant liquid was treated with ethyl ether to yield a second fraction. The extraction process was repeated with an additional 200 ml. of methyl alcohol and the fractions were combined. The white solids of both fractions were tested with ninhydrin reagent and were found to contain free amine. They both gave positive tests for free sulfate and melted over a wide range of temperature, beginning at 210°C. Thus, the product of this reaction was very impure.
IV. DISCUSSION OF RESULTS

A. Purification of Sodium Chondroitinsulfate and the Preparation of Its Salts

1-2. The Preparation of Purified Sodium Chondroitinsulfate and of Sodium Acid Chondroitinsulfate:—The crude sodium chondroitinsulfate used in this investigation was obtained from the Wilson Laboratories, Chicago, Illinois. This crude preparation probably was prepared by extraction of bovine cartilage with dilute alkali followed by removal of protein with kaolin and precipitation with glacial acetic acid. Meyer in his investigation of the various chondroitinsulfuric acids present in tissues stated that the chondroitinsulfuric acid found in cartilage was chondroitinsulfuric acid A; \[ a/D = -30^\circ \] (76). He further stated that so designated chondroitinsulfuric acid was found only in cartilage and was hydrolyzed by testicular hyaluronidase but not by pneumococcal hyaluronidase. This is the material the above cited worker considered to be the most common form. Further, Meyer in a personal communication with this laboratory has stated that the material utilized in this work is the common chondroitinsulfuric acid A.

Because of the above probable precipitation of crude chondroitinsulfuric acid from glacial acetic acid by the manufacturer, Madison considered this material to be sodium.
acid chondroitinsulfate (50). Recent studies of new lots of this material, with regard to methods of purification, indicate that it is sodium chondroitinsulfate (neutral salt).

In early purification of this material, the method of Madison (50) was followed in preparing the sodium salt. This procedure gave a pure product in good yield when the starting material was relatively pure. One minor improvement was noted in the above cited procedure. Madison poured the chondroitinsulfuric acid solution into several volumes of 95 per cent ethanol. Much better results were obtained by adding the alcohol slowly to the aqueous chondroitinsulfate. The tendency for the white flocculent precipitate to collapse into a sirup was decreased many fold. Further, it was found that a mixture of methanol and 95 per cent ethanol gave a still better precipitation. A small amount of saturated sodium chloride solution, added at the point of turbidity, greatly enhanced flocculation with no apparent sacrifice in purity of the final product.

Later shipments of sodium chondroitinsulfate from the Wilson Laboratories contained much greater quantities of protein-like and degraded materials. This crude sodium chondroitinsulfate gave an extremely dark solution. Purification by the usual procedure gave a product which still contained degraded material and which gave positive
tests for protein, free amine and reducing material. This product was definitely unsuitable for use as the starting material for deacetylation and sulfation studies. Therefore, a process was needed for the preparation of relatively pure sodium chondroitinsulfate in rather large quantities. It was desired that this process be rather rapid and thorough. After a rather lengthy study it was noted that a mixture of Magnesol (Westvaco Chemical Corp., South Charleston, W. Va.) and Celite tended to adsorb preferentially the colored material from dark chondroitinsulfate solutions. Studies were conducted in which 10 per cent aqueous sodium chondroitinsulfate solutions were chromatographed on a column packed with Magnesol/Celite (5/1). A dark band developed on the column and collection of the solution passing on through the column yielded a light lemon yellow solution. This solution was processed as described in the experimental section to yield pure sodium chondroitinsulfate. This material was carefully dried prior to elemental analysis and it was shown that the material contained water of hydration proportional to the sulfate content (simple ratio) in agreement with the findings of K. H. Meyer and coworkers as well as those of Mathews and Dorfman (57,74). The material was found to contain approximately 0.75 sulfate groups per disaccharide repeating unit. Other elemental analytical data were
in agreement with the proposed repeating unit of
\( \text{C}_{14} \text{H}_{19} \text{NO}_{11} \text{Na(SO}_3 \text{Na)} \text{2H}_2 \text{O)} \cdot 0.745 \)

Elution of the above-described column yielded a dark material which apparently was degraded chondroitin-sulfuric acid with some occluded protein.

The sodium acid salt of chondroitin-sulfuric acid was easily prepared by precipitation of the above-described neutral sodium salt from a water solution by the addition of glacial acetic acid. After processing as described in the experimental section a product was obtained whose analysis showed it to be pure sodium acid chondroitinsulfate (hydrate). However, that the chromatographic step was essential in the purification was indicated by the fact that acetic acid precipitation did not yield a more pure product. The optical rotations of the neutral sodium salt and the acid sodium salt of chondroitin-sulfuric acid were in good agreement with those cited in the literature for these respective materials. The respective values, uncorrected for water of hydration, were in good agreement with the rule of optical rotation applicable to the free acid and the salt of the free acid. The optical rotations for the neutral sodium salt and the sodium acid salt of chondroitin-sulfuric acid discussed here were \( \alpha^{25D} \text{ -25.08°} \) (c 1.46, H\(_2\)O) and \( \alpha^{24D} \text{ -15.4°} \) (c 1.46, H\(_2\)O) respectively.
That hydrate formation was feasible was supported by the fact, other than analysis of the above, that the disodium salt of 2-sulfaminoethylsulfuric acid formed a stable hydrate (trihydrate), as does sodium sulfate.

In conclusion, it should be stated that pure sodium chondroitinsulfuric acid has been prepared from the crude material by a simple but effective chromatographic procedure. Conversion of the crude material to the sodium acid salt by precipitation with glacial acetic acid followed by conversion back to the neutral sodium salt was found to be unnecessary. The procedure of Madison (50) was found to be effective using the same crude material which that worker had employed.

3. The Preparation of Calcium Chondroitinsulfate:—

In a survey of methods of purification of chondroitin-sulfuric acid, the procedure of Einbinder and Schubert (71) was noted. In this procedure these workers reported the isolation of crystalline calcium chondroitinsulfate. Therefore, experiments were conducted in this laboratory using a modification of the above cited procedure.

An efficient, practical purification procedure was desired. The present work required large quantities of pure chondroitinsulfuric acid as a starting material. This necessitated the use of the crude chondroitin-sulfate, purchased from the previously named source, as the ultimate starting material. This fact alone limited
the possibility of obtaining crystalline chondroitin-
sulfuric acid since the above cited workers prepared
their chondroitinsulfuric acid, prior to crystalliza-
tion, by a method other than alkali extraction. The
starting material in these crystallization experiments
was purified by the chromatographic procedure discussed
earlier (Sec. III-A). This purified sodium salt was
found to be of reasonable purity.

In carrying out the experiments it was found that
kaolin was very difficult to remove from aqueous chon-
droitinsulfate solutions. The turbid solutions thus
obtained required much time in clarification. Therefore,
this process was omitted for practical reasons.

This worker was able to separate calcium chondro.it-
insulfate into three so-called fractions. Two of these
fractions were analyzed and little differences were found
in the elemental chemical constitution of these. However,
some difference was noted in the optical activities of
the respective fractions. The optical rotations for the
mid and tail fractions were $\alpha - \gamma 21D -26.2^\circ$ (c 1.81, H$_2$O)
and $\alpha - \gamma 21D -22.9^\circ$ (c, 1.99, H$_2$O) respectively. Both
fractions gave colorless aqueous solutions and on drying
yielded very white powders. However, neither fraction
was crystalline. All attempts to induce crystalliza-
tion failed. A fair evaluation of this purification
technique would require that it be adhered to completely
since the method of extraction of the chondroitinsulfuric acid from cartilage may be very important.

B. N-Deacetylation of Chondroitinsulfuric Acid

1. The Sodium Methoxide Method: Attempts were made to N-deacetylate sodium chondroitinsulfate by means of ester exchange using sodium methoxide as the catalyst. Any methyl acetate formed was removed by distillation. Several experiments were conducted varying the general conditions of the reaction. The general procedure of Whistler and Jeanes was used (112). However, the reaction was unsuccessful in that only about 15 per cent of the total acetyl was removed.

The failure of this reaction may be attributed to two factors. Firstly, the reaction was heterogeneous. Secondly, the N-acetyl bond of the polymer was stronger than the O-acetyl bond of the formed methyl acetate. Thus the reaction was too far to the left in the following equation to be practical.

\[
\begin{align*}
\text{R-N-O} & \text{-CH}_2\text{CH}_3\text{O} \quad \text{R-NH} + \text{CH}_3\text{-}0\text{-C-CH}_3 \\
\end{align*}
\]

2. Acetyl Analysis: The N-acetyl analysis employed throughout this work was a modification of the Freudenberg procedure (2) as employed by Wolfman, Weisblat, Karabinos, McNeely and McLean (18).
This procedure gave reproducible results provided certain precautions were taken. The liquid level in the boiling flask had to be maintained at a constant level of 50 ml. volume. The rate of distillation had to be such that no bumping occurred. Too rapid a distillation rate invariably led to high results. The rate maintained in this analysis was about 1 ml./minute. At least 300 ml. of distillate was collected in 200 ml. portions with each determination. The latter was necessary for an accurate blank correction. In studying the rate of distillation of the liberated acetic acid, it was found that about 80 per cent was distilled over in the first 200 ml. of distillate. After the distillation of 600 ml. of liquid the titration of succeeding 200 ml. aliquots with dilute alkali was a constant. Further, it was found that each batch of p-toluenesulfonic acid required a new blank. This compound was slowly hydrolyzed during the analysis and led to the distillation of some acidity (corrected for in the blank). It was found imperative that each 200 ml. aliquot be heated to boiling before titrating with dilute alkali to a phenolphthalein end point. This step assured the removal of all entrained carbon dioxide by boiling the acid solution before titration.

3. The Cuprammonium Hydroxide Method:—The deacetylation of sodium chondroitinsulfate by means of concentrated
cuprammonium hydroxide was not successful. A typical reaction, which was conducted for 3 weeks, yielded a product which contained little or no free amino function. This reaction was conducted at room temperature in the presence of copper catalyst, adding as a chelating agent for any free amine formed, in the presence of a tremendous excess of ammonium hydroxide (29% NH₃ assay). It was concluded that ammonium hydroxide was not basic enough to promote the reaction.

4. Method Employing Dilute Sodium Hydroxide:--Deacetylation of sodium chondroitinsulfate employing dilute sodium hydroxide was not successful. Mild conditions of temperature and time resulted in a product which was deacetylated to an extent of about 10 per cent. Higher temperatures resulted in the destruction of the material due to hydrolysis in the basic medium.

5. The Deacetylation of Sodium Chondroitinsulfate Using Concentrated Sodium Hydroxide:--The partial deacetylation of chitin using concentrated sodium hydroxide solution was noted (118). The procedure called for the


treatment of insoluble chitin with concentrated alkali at elevated temperatures for long periods of time. This
process effectively removed 85 per cent of the N-acetyl of chitin and gave as a final product a polymer.

The conditions of this reaction were considered much too drastic for the much more sensitive sodium chondroitinsulfate. However, unlike chitin, sodium chondroitinsulfate was appreciably soluble in 40-50 per cent aqueous sodium hydroxide. It is a well known fact that homogeneous reactions proceed under milder conditions than heterogeneous ones. Therefore, it was considered possible to N-deacetylate the material under milder conditions. A lower reaction temperature was considered the most important.

In the presence of concentrated sodium hydroxide, sodium chondroitinsulfate was subjected to two undesir­able side reactions which lead to depolymerization of the polymer. They were basic hydrolysis of the glycosidic bonds and oxidative degradation in the presence of the alkali. It was found that the addition of antioxidants (benzyl alcohol and sodium sulfite) to the reaction mixture and the removal of all the oxygen present by means of a nitrogen atmosphere suppressed the latter reaction. Concentrated alkali possibly tended to minimize the former reaction. It was found that more dilute sodium hydroxide solutions rapidly became dark in color. The most criti­cal consideration in this reaction was the elimination of all oxygen from the alkaline chondroitinsulfate solu­
Darkening of the reaction mixture was definitely suppressed by the addition of the above named antioxidants. These antioxidants apparently suppressed degradation provided they were added before or concurrently with the sodium chondroitinsulfate solution. The benzyl alcohol tended to form an emulsion with the alkali and darkened during the reaction. It was found advisable to recover the partially N-deacetylated chondroitinsulfate as quickly as possible after the completion of the reaction. Extension of the reaction time to 96 hours led to a product in less yield, which had a lower carbon-nitrogen ratio. This tended to indicate that at a certain time in the reaction the desired product began to be destroyed faster than it was being prepared. The reaction thus appeared to have its limitations.

Sodium chondroitinsulfate was partially N-deacetylated by reaction with 50 per cent sodium hydroxide solution for a period of 48 hours at room temperature. This reaction, as conducted in a nitrogen atmosphere in the presence of sodium sulfite and benzyl alcohol, led to the isolation of two products by differences in water solubility. A minor highly N-deacetylated fraction was isolated which had an acetyl content of 2.29 per cent. A major component was isolated which had an acetyl content of about 3.5 per cent. These determinations were made on purified but not dialyzed partially N-deacetylated
sodium chondroitinsulfate. The minor component was found to be very unstable and of low molecular weight since it was almost completely lost on dialysis for 2 days against distilled water in a cellulose membrane which retained particles of molecules weighing 8000 or greater. Dialysis of the major component led to retention of approximately 67.5 per cent of the material. Before dialysis this major component had an acetyl content of approximately 0.4 acetyl groups per disaccharide unit, while after dialysis this material apparently contained 0.6 acetyl groups per disaccharide unit as confirmed by elemental analysis of this material and acetyl analysis of its sulfation derivative as well as elemental analysis. Elemental analysis (C, H, Na) of this final major component indicated a disaccharide repeating unit, for this polymer, with an empirical formula of
\[ \text{C}_{12}\text{H}_{16.78}\text{Na}(\text{SO}_{3}\text{Na} \cdot 2\text{H}_{2}\text{O})_{0.62}(\text{C}_{2}\text{H}_{3}\text{O})_{0.6} \] and a molecular weight of 472.2. During its strong alkali treatment the sodium chondroitinsulfate had been desulfated to an extent such that only about 0.62 sulfate groups remained per disaccharide repeating unit. The optical rotation of this final product was \( [\alpha]_{D}^{23} = -20.3^\circ \) \( (c 1.97, \text{H}_{2}\text{O}) \).

In the course of several such preparations no attempt was made to separate the partially N-deacetylated sodium chondroitinsulfate into two fractions because the
sulfation derivative of this material was the final desired product. Thus any low molecular weight material was lost in the dialysis of the sulfation product.

Partially N-deacetylated sodium chondroitinsulfate, as prepared here, gave a positive test for the free amino group, and positive tests for hexosamine and hexuronic acid. Bound sulfate was found present in this material.

6. The N-Deacetylation of Chondroitinsulfuric Acid by Amide Exchange:- The N-deacetylation of chondroitin-sulfate by means of concentrated sodium hydroxide was successful in that a certain degree of N-deacetylation was obtained and a product was obtained in a fair yield. However, a milder procedure was desired in which a higher degree of N-deacetylation was obtained with a minimum amount of degradation of the polymer. Application of the well known ester-exchange procedure utilizing sodium methoxide was unsuccessful. This reaction suffered from the fact that equilibrium in the following equation was too far to the left because the $\text{-C-N-}$ bond of the amide was much stronger than the $\text{-O-O-}$ bond of the ester.

$$\text{CH}_3\text{O}^- + R - \text{O} \text{-} \text{N} \text{-} R' \underset{\text{R} - \text{O} - \text{CH}_3 + R' - \text{NH}^-}{\xrightarrow{\text{R}'} \text{R} \text{-} \text{N} \text{-} \text{H} + \text{CH}_3\text{OH}}$$

Also, the reaction was necessarily heterogeneous.
The application of amide exchange to the N-deacetylation of sodium chondroitinsulfate was considered possible since the amide formed in the following reactions should have about the same bond strength as the N-acetyl bond of the polymer.

\[
\begin{align*}
R - \text{NH} + R' - N - C - R'' & \overset{\text{R - N - C - R''}}{\underset{\text{R' - N: -}}{\rightleftharpoons}} R - \text{N: -} + R' - \text{NH}_2 \\
\end{align*}
\]

However, it was felt that two requirements had to be met in the reaction. Firstly, the reaction had to be homogeneous so as to eliminate high reaction temperatures. Secondly, the amine base used in the reaction had to be a strong base so as to shift the reaction to the right in the limiting step.

The amino base chosen for this reaction was ethanolamine. This was chosen for two reasons. Firstly, it is a strong base. Secondly, the amide of ethanolamine (N-acetylethanolamine) has been found to be soluble in non-polar solvents. Thus the advantages of liquid-liquid extraction could be applied to the reaction. It was felt that this technique would shift further the reaction to the right. Formamide was chosen as a solvent for the reaction because both ethanolamine and sodium chondroitinsulfate are soluble. Also, formamide is a polar solvent and the reaction product, N-acetylethanolamine,
should have been more soluble in a non-polar extracting solvent than in the reaction medium. Thus it should have been possible to remove \( \text{N-} \)acetylethanolamine almost as fast as it was formed. Also, formamide should have reacted with the deacetylating agent since it too is an amide. Deformylation of the formamide should have been accompanied by the evolution of ammonia. Ammonia evolution should have been an indication that amide exchange was taking place. A few drops of concentrated hydrochloric acid were added to the reaction mixture as a catalyst. It has been shown that in the deamidation of the amide of the Gabriel reaction with hydrazine, small amounts of acid catalyze the reaction. Any traces of water, introduced with the hydrochloric acid, were removed by liquid-liquid extraction with ethyl ether since water should have accumulated in the side arm flask. This was followed by extraction with toluene in which \( \text{N-} \)acetylethanolamine has been found to be soluble (as with ethyl ether).

Application of the reaction outlined above apparently was successful. A product was obtained in 85 per cent yield which gave an excellent test (ninhydrin) for the free amino group and which apparently was 76 per cent deacetylated. Acetyl determinations were made using a modification of the Freudenberg procedure (20). The isolated product was a white powder. It showed varia-
tions in solubility in water according to the pH of the solution, tending to be insoluble in basic medium. The material was essentially non-reducing and contained bound sulfate. It gave positive hexosamine and uronic acid tests. The material was submitted for elemental analysis and these agreed with both the calculated values and the acetyl determinations. Elemental analysis of this product was in agreement with a disaccharide repeating unit with a molecular weight of 458.3 and an empirical formula of \( \text{C}_{126}\text{H}_{16}\text{N}_{10}\text{Na}(\text{SO}_{4}\text{NaH}_{2}0)_{0.745} \) \( (\text{C}_{2}\text{H}_{3}0)_{0.234} \). This product had an optical rotation of \( [\alpha]^{256}D -22.1^\circ (c 0.972, \text{H}_{2}0) \).

Some difficulty was encountered in removing formic acid from the deacetylated product. Neutralization led to the formation of sodium formate which had similar solubility characteristics as the product. Ammonia was given off during much of the reaction as a further indication that the reaction was proceeding.

One further point should be mentioned. Due to the nature of the reaction there was a possibility that any free amino formed in the chondroitinsulfate might have been esterified by formate ions. In one instance sodium formate was isolated from an aqueous solution of the desired N-deacetylated chondroitinsulfate. It was found that all reactants in this reaction had to be pure and
that adherence to the reaction sequence discussed in the experimental section was essential.

C. Sulfation of N-Deacetylated Chondroitinsulfuric Acid and Properties of the Product

1. Employing Pyridine and Chlorosulfonic Acid:

Partially N-deacetylated sodium chondroitinsulfate has been sulfated by a modification of the procedure of Astrup, Galma and Volkert (90). Sulfation of the material with chlorosulfonic acid in pyridine at 90°C, followed by dialysis and recovery led to the isolation (as the sodium salt) of a product in 70-90 per cent yield which was a light tan water-soluble powder with an optical rotation of $\alpha_{D}^{27} = -19.9^\circ$ (c 2, H$_2$O). This sulfated product contained bound sulfate, hexosamine, uronic acid, was non-reducing to Benedict's solution and contained no free amino group. A typical analysis of this material showed it to contain sulfur corresponding to 3.55 sulfate groups per disaccharide unit and an acetyl content corresponding to 0.59 acetyl groups per disaccharide unit. Elemental analysis was in agreement with the above findings and was indicative of a disaccharide repeating unit of molecular weight 746.6.

Chemical evidence strongly indicated that the free amino function originally present in the N-deacetylated starting material was blocked in an acid-hydrolyzable
sulfamate linkage. Infrared studies of this and similar materials supported these chemical findings.

One of the more important factors in obtaining the above sulfation results was the activation of the partially N-deacetylated sodium chondroitinsulfate in dry pyridine prior to sulfation. This activation procedure was described in the experimental procedure and permitted the reaction to proceed under milder conditions than if the dry powder were sulfated directly.

In vitro anticoagulant assays were conducted with this material and it was found to have an activity of 57 International Units per milligram as compared to a value of 110 International Units per milligram for standard heparin. In direct contrast sodium chondroitininsulfate, which had not been partially N-deacetylated prior to sulfation, possessed an anticoagulant activity of less than 10 International Units per milligram. This further substantiated the importance of the N-sulfate group in the anticoagulant activity of these materials.

Clinical studies of this partially N-deacetylated and sulfated sodium chondroitininsulfate have shown that its prolongation of clotting of fresh rabbit and dog blood was not as great as that of standard heparin. Further in vivo studies of the relative toxicity of this material in mice as compared with heparin have shown that the two are of the same magnitude and type. In
conclusion, this sulfated derivative of partially N-
decacylated sodium chondroitinsulfate was found to
contain roughly one-half as much N-sulfate as heparin
per disaccharide unit and was found to have about one-
half the in vitro anticoagulant activity of heparin.

2. Homogeneous Sulfation in Formamide:—A relatively
mild procedure was desired for the sulfation of N-
decacylated sodium chondroitinsulfate. The procedure
in use in this laboratory required a rather high reaction
temperature. The product of this latter sulfation pro-
cedure, while possessing a good anticoagulant activity,
was somewhat colored and yielded a dark solution on
dissolving in water. That degradation occurred during
the reaction was evidenced by the above facts and also
by the fact that some gas was evolved during the reac-
tion. It was felt that less degradation of the polymer
during sulfation might lead to a product of higher anti-
coagulant activity. The pyridine-chlorosulfonic acid
procedure was essentially heterogeneous.

The most apparent solution to this problem was in
the development of a homogeneous sulfation procedure
which would permit a lowering of the reaction tempera-
ture. The sulfation of chondroitinsulfuric acid in
formamide solvent was noted (111). However, the above
cited procedure had two undesirable aspects. Firstly,
it was believed that the acidity of a solution of chlorosulfonic acid in formamide was very great and secondly, the isolation of the free sulfamic acid type compound, without conversion to the neutral sodium salt, had been found to be unsuccessful with this polymer as well as heparin. Great acidity would have led to polymer degradation and the evolution of quantities of hydrogen cyanide gas (decomposition of formamide). Previous work concerned with the acetylation of sodium chondroitinsulfate had shown the feasibility of employing a pyridine-formamide solvent. It was thought that this would accomplish two objectives. Firstly, the reaction would be homogeneous and secondly, the acidity of the solution would not be nearly so great because of salt formation between the chlorosulfonic acid and the pyridine. It was also considered advisable to convert the sulfated product to its sodium salt by means of sodium methoxide so as to eliminate hydrolysis in an aqueous medium.

The homogeneous sulfation reaction was carried out over a period of 24 hours and the product was neutralized with sodium methoxide. A very light colored product, which also yielded a light colored solution in water, was obtained in about 95 per cent yield. This reaction, conducted at room temperature, yielded a product which had a total sulfur content of 14.97 per cent and an
optical rotation of $\left[\alpha\right]_{D}^{24} = -18.6^\circ$ (c 1.55, H$_2$O). This degree of sulfation was almost identical with that obtained by sulfating the same starting material under the more drastic heterogeneous sulfating conditions. The anticoagulant activities of the two products were comparable (40-50 per cent of that of heparin) and seemingly limited in magnitude by the degree of N-deacetylation of the sodium chondroitinsulfate. The product of this reaction contained bound sulfate, hexosamine, and uronic acid residues. The material gave a negative test for the free amino group and was extremely water-soluble.

The homogeneous procedure was found to be much more practical than the previous procedure in that no tedious activation of the N-deacetylated chondroitinsulfate in pyridine prior to sulfation was necessary. Further, the apparently less degraded product of the homogeneous sulfation procedure yielded a lighter colored aqueous solution necessary for accurate determination of the optical activity of the material. Under the conditions of the experiment apparently no hydrogen cyanide gas was evolved.

The only disadvantage of the procedure seemed to be that on neutralizing the sulfation product with sodium methoxide the polymer precipitated from the solvent medium before neutralization was complete. This led to
the isolation of a sodium acid derivative which possessed some of the instability characteristic of sodium acid heparinate. However, the above situation may be easily eliminated.

Clinical evaluation of this material has, as yet, not been conducted. However, this material should be less toxic than that sulfated material prepared by the chlorosulfonic acid-pyridine procedure since the material has been processed under milder reaction conditions. These should have led to less degradation of its polymer and less interaction with pyridine.

D. The Acetylation of Sodium Chondroitinsulfate.

It was desired to completely acetylate chondroitinsulfate without simultaneous desulfation of the material. It also was desired to obtain a product which was not substantially degraded. This derivative was desired for future structure studies with regard to the allocation of the position of the sulfate group in the polymer as well as the nature of the glycosidic linkages present. This derivative was of interest in connection with infrared studies of chondroitinsulfate derivatives. The O-acetyl groups should have given a definite absorption maximum in the infrared spectrogram as compared to the starting material. Also it was hoped that a completely acetylated chondroitinsulfate
would produce an infrared spectrogram with a minimum of interference from hydroxyl group absorptions. Finally it was thought that this compound might have solubilities such that different deacetylation techniques could be applied as compared to the starting material.

The complete acetylation of desulfated, acid-degraded chondroitinsulfate was described by M. L. Wolfrom and Rex Montgomery (27) as well as by Stacey and associates (52). The acetylated chondroitin obtained by Stacey was highly degraded. That of Wolfrom and Montgomery also was highly degraded. The latter workers obtained a compound, using acetic anhydride in absolute sulfuric acid, which was reducing, passed through a cellulose membrane on dialysis, contained no sulfate, and had an acetyl content which was somewhat high for the high molecular weight polymer. These workers necessarily used rather drastic acetylating conditions.

The use of formamide as an acetylation solvent for pectin (119) and hyaluronic acid (120) in conjunction with pyridine and acetic anhydride, was noted. However, it was found that unlike the above materials, sodium chondroitinsulfate was soluble in dry formamide. It
was also found that an equal volume of dry pyridine could be added to this solution without causing the chondroitinsulfate to precipitate. However, additional pyridine caused precipitation to occur. Immediately, it was realized that milder acetylation conditions could be employed than was the case with pectin and hyaluronic acid. The latter materials were acetylated heterogeneously in a swollen state.

Experiments were conducted employing a six-fold by weight excess of acetic anhydride to sodium chondroitinsulfate, considering three free hydroxyl groups per disaccharide unit. One acetylation was run 1 week at 0-5°C. The reaction yielded a product which contained about 24 per cent total acetyl. Reaction for 24 hours at room temperature yielded a product containing about 28.0 per cent total acetyl. This was the desired product. The exact proportions of reactants and solvents found to be the most suitable are given in the experimental section.

The reaction was found to go very smoothly if certain precautions were taken. All reactants and the solvent had to be absolutely dry. Any moisture introduced at any time during the reaction led to a darkening of the solution and a product low in acetyl content.

The product, isolated first by alcohol precipitation from the acetylation solution, was a white powder.
However, on redissolving in water it was found that precipitation by the addition of absolute ethanol was unsatisfactory in that the small amount of sodium chloride added to bring about coagulation was less soluble in the water-ethanol solution than the acetylated chondroitin-sulfate. Precipitation by this method led to a product contaminated with sodium chloride. The best method of final purification, after alcohol precipitation, was found to be dialysis followed by lyophilization to yield a white fluffy material.

The final product was insoluble in chloroform, ether, absolute methanol and absolute ethanol but was soluble in water, formamide and 1:1 water-alcohol solution. Unlike the product of previous workers, this material was non-reducing to Benedict's solution and contained all the bound sulfate originally present in the starting material. It gave positive tests for uronic acid residues and hexosamine residues. After dialysis in a membrane calibrated to retain materials of molecular weight greater than 8000 the overall yield was 85 percent of the value calculated for a product containing four acetyl groups per disaccharide unit. Therefore, it was substantially undegraded. The water-solubility of this material was remarkable.

This fully acetylated material showed a property which was rather unique and may have further application.
Treatment of an aqueous solution of this material with sodium iodide and barium iodide led to the release of sulfate after two days at room temperature although it was shown to contain bound sulfate by the standard qualitative test using aqueous barium chloride and hydrochloric acid. Further, the material gave a negative ninhydrin test. An infrared spectrogram of this material gave a very distinct O-acetyl peak and produced a sharpening of the rather broad O-sulfate peak. Also present in the spectrogram were absorption peaks present in the infrared spectra of sodium chondroitinsulfate. This shall be discussed further in the appropriate section of this dissertation.

This acetylation reaction was further substantiated, and in turn supported, the findings with regard to the purification of sodium chondroitinsulfate. A sample of sodium chondroitinsulfate, purified only by chromatography and alcohol precipitation, yielded a product which contained approximately four acetate groups per disaccharide unit. The carbon, hydrogen, nitrogen, sulfur and ash ratios in the basic repeating unit remained essentially the same as the starting material. The quantitative analysis of this product and that obtained by R. K. Madison (50) are given in Tables X and XI. The optical rotation of this material was found to be $[\alpha]_21^\text{D} -27.85$ (c 1.63, H$_2$O).
Table X

Analysis of Acetylated Chondroitinsulfate, Disodium Salt

<table>
<thead>
<tr>
<th>Found (a)</th>
<th>Required for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C12H13NO10 Na(G2H3O)4 SO3Na2H2O(*)</td>
</tr>
<tr>
<td>I (b)</td>
<td>D</td>
</tr>
<tr>
<td>II (c)</td>
<td>D</td>
</tr>
<tr>
<td>C</td>
<td>38.41D</td>
</tr>
<tr>
<td>H</td>
<td>4.91D</td>
</tr>
<tr>
<td>N</td>
<td>2.44D</td>
</tr>
<tr>
<td>Na</td>
<td>5.94D</td>
</tr>
<tr>
<td>Total Acetyl</td>
<td>26.90</td>
</tr>
</tbody>
</table>

* The disodium salt of a disaccharide repeating unit containing four acetyl groups and three-fourths of a sulfate group.

(a) Drying conditions are as specified in Sec. III-A
(b) Preparation I
(c) Preparation II
D Departmental analyst, W. H. Deebel.
H Huffman Microanalytical Laboratories, Wheatridge, Colorado.
Table XI


<table>
<thead>
<tr>
<th>Found</th>
<th>Required for C_{44}H_{57}O_{30}N_{2}Na(a)</th>
<th>Required for C_{68}H_{91}O_{44}N_{4}Na (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>47.54, 47.22</td>
<td>47.50</td>
</tr>
<tr>
<td>H</td>
<td>5.49, 5.47</td>
<td>5.11</td>
</tr>
<tr>
<td>N</td>
<td>2.56, 2.46</td>
<td>2.51</td>
</tr>
<tr>
<td>Ash (Na_{2}SO_{4})</td>
<td>6.13</td>
<td>6.36</td>
</tr>
<tr>
<td>O-acetyl</td>
<td>37.8</td>
<td>30.8</td>
</tr>
</tbody>
</table>

(a) The acid salt of a tetraacetate unit composed of 2 hexosamine residues and 2 glucuronic acids.
(b) The acid salt of a hexosaccharide unit composed of four hexosamine residues and two glucuronic acids.
E. The Preparation of Ethanolamine Disulfate

The synthesis of the disulfate of 2-aminolthanol was desired. This compound was of interest because it was the simplest possible organic compound containing both an O-sulfate group and a sulfamic acid group. Therefore, it was a very desirable compound for hydrolysis rate studies and infrared data. p-Aminoethyl sulfuric acid ethanolamine is a commercially available compound. However, there is some question as to whether the sulfate group is on the amino group or the hydroxyl group (XIX, XX). This situation is more complicated in aqueous solution because of the possible migration of the sulfate group between the two positions. And indeed, this phenomenon may occur in the deactivation of heparin. An hydrolysis study of this compound was considered of little value because the question stated above would have made the results indefinite with regard to application to the hydrolysis of N-sulfate and O-sulfate groups. The preparation of the disulfate (XXI) was considered a solution to this problem.

\[
\begin{align*}
\text{HN}_3^+\text{--CH}_2\text{--CH}_2\text{--OSO}_2\text{O}^- & \quad \text{HO--SO}_2\text{--NH--CH}_2\text{--CH}_2\text{OH} \\
\text{XIX} & \quad \text{XX} \\
\text{Na}_2\text{S--N--CH}_2\text{--CH}_2\text{--OSO}_3\text{Na} & \quad \text{XXI}
\end{align*}
\]
Wenker synthesized β-aminoethylsulfuric acid by treating ethanolamine with 96 per cent sulfuric acid (121). The synthesis was improved by Leighton, Perkins, and Rinquist (122). Rollins and Calderwood (123-124) prepared this compound using fuming sulfuric acid and ethanolamine. In the two cited papers they described the chemical properties of this compound. They showed that it did not contain a free amino group and did not react with benzoyl chloride. This compound consumed two equivalents of base and was slowly hydrolyzed by aqueous hydrochloric acid. These workers considered the compound to be a Zwitterion. Further, they stated that it might possess a cyclic structure or be stabilized by ring-chain isomerism in which the ring structure predominated. Therefore, rather than become involved in this interesting case of sulfate migration, it was decided to synthesize the fully sulfated compound because there would be no doubt as to the location of the sulfate groups.
in such a system. It also was desired to avoid all moisture in the reaction because of the possible instability of a so located sulfamic acid group. It was felt that moisture might provide conditions by which one of the sulfate groups would be expelled by a back-sided attack of the other acidic sulfate group on the next carbon atom. However, it was felt that if the compound could be converted to the disodium salt it would then be stable.

Two general sulfation methods were employed in this work. The reaction of chlorosulfonic acid and pyridine with ethanolamine first was studied. This led to the isolation of a mixture of salts which could not be separated. However, the reaction of sulfur trioxide and pyridine was used successfully.

The reaction of sulfur trioxide in pyridine with purified ethanolamine produced the disulfate of ethanolamine. This compound was isolated first as the disodium salt and then purified as the crystalline trihydrate of the disodium salt. This hydrate was stable and gave a sharp melting point. It gave a positive test for bound sulfate but a negative test for a free amino function. Exactly three molecules of water and two sulfate groups were present per ethanolamine residue. An x-ray powder diagram was made of the compound and this confirmed the crystallinity of the material. The compound produced
an infrared spectrum very similar to that of sulfamic acid but containing additional absorption peaks indicative of O-sulfate and water. As will be seen in a later discussion, this model compound was the one needed to establish the location of the infrared absorption of the sulfamic acid group in the sulfated polysaccharides now under study. This compound was a good intermediate compound for correlating the above polysaccharides with the infrared spectrum of sulfamic acid itself. The melting point of the product was 220-221°C. (uncorr.). An analysis of this product is given in the experimental section. The only formula that could possibly represent those results is given below.

\[
\begin{align*}
&H \\
NaO_3S - N - CH_2 - CH_2 - OSO_3Na \\
&\quad \text{• } 3H_2O
\end{align*}
\]

\[XXII\]

F. Infrared Spectra of Sodium Chondroitinsulfate and Related Materials

It was considered of interest to prepare infrared spectrograms of the various sodium chondroitinsulfate derivatives prepared in this laboratory as well as those of heparin, sulfated chitin and a few sulfamic acid derivatives. It was hoped that a comparison of this infrared data might yield some qualitative evidence which would further substantiate the importance of the \( R-H-\text{SO}_3\text{Na} \)
group in the anticoagulant activity of these sulfated materials. In particular, a purely qualitative identification of the absorption region of the N-SO$_3$Na or sulfamic acid group was desired. It was felt that if evidence indicative of such a relationship were found a more exacting investigation could be undertaken in the future. Therefore, any information presented in this discussion should be considered as supporting evidence and not as absolute proof of structural relationships of the compounds under consideration. Furthermore, no attempt has been made to present a complete review of the field of infrared spectroscopy in this brief discussion. However, some of the literature concerning the infrared spectroscopy of compounds closely related to those under consideration will be presented.

Orr, Harris and Sylven (125) prepared infrared spectrograms of hyaluronic acid, sodium hyaluronate, chondroitinsulfuric acid and sulfate esterified hyaluronic acid. These workers obtained the spectra on a Perkin-Elmer 12C spectrometer with a rocksalt prism, using films prepared by casting the polymers from aqueous solutions.

Concerning the spectra of hyaluronic acid, they
assigned the absorption band at 1,735 cm\(^{-1}\) to an unionized carboxyl group and the pair of bands at 1,648 and 1,560 cm\(^{-1}\) to a monosubstituted amide group. In the spectrum of the sodium salt of this material they noted that the free carboxyl band had disappeared and bands at 1,610 and 1,410 cm\(^{-1}\), characteristic of the carboxyl ion had appeared. They noted some overlapping of amide bands but separated them graphically. These workers noted the absence of an absorption band at 1,735 cm\(^{-1}\), characteristic of acetylated hydroxyl groups.

With regard to the spectra of chondroitinsulfuric acid and sulfate-esterified hyaluronic acid, these workers noted a strong absorption band, with a broad maxima, between 1,230 and 1,255 cm\(^{-1}\). This they assigned to the O-sulfate group.

The assignment of the band at 1,735 cm\(^{-1}\) to that of an unionized carboxyl and a pair of bands at 1,610 and 1,410 cm\(^{-1}\) to the carboxylate ion was in agreement with the findings of Klotz and Gruen (126) as well as those of Gore, Barnes and Petersen (127) in their study of the


infrared spectra of certain organic acids and their sodium salts. Richards and Thompson (128) had assigned previously absorption bands in the region of 1,648 and 1,560 cm\(^{-1}\) to that of the amide function. Klotz and Gruen (126) assigned a very strong and broad absorption maximum between 1,230 and 1,255 cm\(^{-1}\) to that of the sulfate in sodium alkyl sulfates. The acetate function (O-acetyl) was assigned to an absorption region at 1,470 cm\(^{-1}\) by Thompson and Torkington (129).

Levine, Stevenson and Kabler (130) made a qualitative infrared study of several pneumococcal polysaccharides using a Perkin-Elmer recording spectrophotometer. These workers classified the polysaccharides in accordance with the functional groups found present in each grouping. They utilized amide band absorption bands near 1,560 and 1,660 cm\(^{-1}\) and carboxylate bands near 1,610 and 1,390 cm\(^{-1}\).
In a later publication Orr (131) demonstrated some fundamental differences in chondroitinsulfuric acids isolated from different sources by means of a qualitative identification of functional groups in the various preparations.

He also included hyaluronic acid in this study as well as polysulfated hyaluronic acid and polysulfated alginic acid. He showed that chondroitinsulfuric acid differed from hyaluronic acid mainly in that the former gave an infrared absorption band at about 1,240 cm\(^{-1}\) which was due to the sulfate group. This absorption band appeared in the spectra of polysulfated hyaluronic acid and polysulfated alginic acid confirming the above assignment.

This worker attributed this absorption to \(S=O\) stretching vibration in the molecule analogous to the 1,735 cm\(^{-1}\) \(C=O\) stretching vibration in acetates. This worker also assigned a \(C-O-S\) stretching vibration to a band at about 820 cm\(^{-1}\) in polysulfated hyaluronic acid and to two bands at 824 and 830 cm\(^{-1}\) in polysulfated alginic acid (free) and 802 and 834 cm\(^{-1}\) in the salt of polysulfated alginic acid. However, the material did not contain the N-sulfate grouping.

Kuhn (132) adequately demonstrated the assignment
of the absorption bands of several functional groups in an excellent presentation of the infrared spectrograms of several carbohydrate derivatives. Among others, the carboxyl group was shown to have an absorption band near 1,790 cm$^{-1}$ as found in D-glucuronic acid. The carbonyl band of acetates was demonstrated to be near 1,660 cm$^{-1}$. A number of cellulose derivatives were presented and assignments made for, O-acetyl groups, nitrate groups, carboxyl groups, benzene ring containing groups and aromatic groupings present in these materials.

Recently, Baxter, Cymerman-Craig and Willis (133) have conducted a study of the infrared spectra of a number of aryl sulfonamides. These workers have tentatively assigned an absorption band near 1,070 cm$^{-1}$ to that of the S-N band in these compounds, with reservations due to certain abnormalities occurring as noted in their article. However, some shifting of this band would be expected in the infrared absorption spectra of polysaccharides containing this grouping.

In this attempt to assign an infrared absorption
band to the C-N-SO$_3$Na grouping three model compounds were employed. These were sulfamic acid, ethanolamine disulfate trihydrate and p-ethylaminosulfuric acid. Chondroitinsulfuric acid was considered a model compound also in that a study was made of the changes in the infrared absorption of this material with respect to known chemical changes. Heparin, sulfated chitin, sulfated chondroitinsulfate, sulfamic acid and ethanolamine disulfate were known from chemical evidence to possess the sulfamic acid group. The problem resolved itself into deciding what absorption bands, if any, these materials had in common which likewise were not present in sodium chondroitinsulfate. The infrared spectra of all the materials were obtained from Nujol mulls in a salt cell in a Baird I. R. recording spectrophotometer. However, the infrared spectrum of water was obtained employing an old sodium chloride cell.

The infrared spectrum of sodium chondroitin- sulfate (Fig. 6) was characterized by broad absorption maxima at 1,220-1,250 cm$^{-1}$, 1,560-1,660 cm$^{-1}$ and a maximum at about 3,400 cm$^{-1}$. These bands were in regions previously assigned to O-sulfate, carboxyl and amide, and hydroxyl or amino respectively. A broad maximum centered at about 1,030 cm$^{-1}$ was noted. This band appeared in the three polymers under consideration. The infrared spectrum of acetylated chondroitinsulfate (Figs. 12,13)
showed a very distinct band at 1,740 cm\(^{-1}\) which was characteristic of the O-acetate grouping. Partially N-deacetylated chondroitinsulfate was sulfated under conditions which produced a product in which the amino group was blocked either in a sulfamic acid linkage or residual N-acetyl. The spectrum of this material (Fig. 10) possessed one band not found in the spectrum of sodium chondroitinsulfate. This was a broad band centered at 1,000 cm\(^{-1}\). Comparison of this spectrum with those of heparin (Figs. 7-9) and sulfated chitosan (Fig. 11) showed that all possessed a broad absorption band at 1,000 cm\(^{-1}\).

The disulfate of ethanolamine was prepared. Analysis of this crystalline hydrate showed that one sulfate group had to be present in a sulfamic acid linkage. This overcame the old problem of allocation of the position of the sulfate group in \(\beta\)-aminoethylsulfuric acid. An infrared spectrogram was prepared of this material (Fig. 4) and compared with the spectrum of pure sulfamic acid (Figs. 2,3). Both pure compounds possessed broad absorption bands at 1,250 cm\(^{-1}\) and a rather sharp band at 1,063 cm\(^{-1}\) and a rather sharp band at 1,000 cm\(^{-1}\). The ethanolamine derivative, since it was a hydrate, demonstrated hydroxyl bands elsewhere in the spectrum. The band at 1,000 cm\(^{-1}\) coincided with the band found in the sulfated polymers. As was expected, the absorption
peaks were sharper in the model compounds than in the polymers. The infrared of β-aminoethylsulfuric acid (Fig. 5) showed bands near 1,000 cm⁻¹ indicating some C-N-S bonding in the material. The above evidence tended to indicate that the C-N-SO₂⁻Na grouping showed an absorption band near 1,000 cm⁻¹ if not multiple bands in this region. The infrared spectra of sulfamic acid and 2-sulfaminoethyl sulfuric acid hydrate (ethanolamine disulfate hydrate) were strikingly similar although sulfamic acid did not contain the C-N-S grouping. This evidence strongly inferred that the absorption maxima at 1,000 cm⁻¹ was that of the N-S bond of the sulfamic acid group. That this absorption peak at 1,000 cm⁻¹ was not due to water was shown by the fact that the absorption spectrum of water was linear in this region (Fig. 16). The absorption peaks at 3,550 cm⁻¹ and 1,660 cm⁻¹ in the 2-sulfaminoethylsulfuric acid (Fig. 4) may be assigned to the C-N bond tentatively. The presence of water of hydration in this compound may be demonstrated by peaks near 3,550 cm⁻¹, 2,300 cm⁻¹, 2,100 cm⁻¹ and possibly one near 1,200 cm⁻¹. The absorption peak at 2,450 cm⁻¹ in sulfamic acid (Figs. 2,3) may demonstrate inner salt (Zwitterion) formation in this free acid.

That the sulfamic acid grouping found in these N-sulfated polysaccharides had not been assigned a definite infrared absorption region by other works may be attri-
buted to the lack of model compounds and sulfated polymers necessary for the study. In this particular study the infrared spectrum of 2-sulfaminoethylsulfuric was very effective in relating the spectra of sulfamic acid with the polymers under consideration.

A few minor observations concerning this infrared study should be related at this time. The spectrum of two preparations of acetylated sodium chondroitinsulfate were presented (Figs. 12, 13). That the amount of absorption assigned to the O-acetyl band (1,740 cm\(^{-1}\)) was proportional to the relative amount of O-acetyl present was shown by the fact that Preparation I (Fig. 11) was found by chemical analysis to contain 23 per cent total acetyl while preparation II (Fig. 12) was found to contain only 24 per cent total acetyl. The absorption band near 1,660 cm\(^{-1}\) found common to all of the polymers is possibly due to groupings (other than N-acetyl) found common to these carbohydrate polymers (carboxyl groups, glycosidic linkages, C-N bonds and carboxyl bonds.)

Reference should be made at this time to the sources of the various compounds employed in this study.

Sulfamic acid (Fig. 2) was a standard reference infrared spectrogram prepared and published by Samuel P. Sadler and Sons, Inc., Philadelphia, Pa. Sulfamic acid (Fig. 3) was recrystallized C.P. sulfamic acid
obtained from the G. Frederick Smith Co., Columbus, Ohio.
2-Sulfaminoethyisulfuric acid trihydrate (disodium salt),
(Fig. 4), prepared by this worker as described in Sec. III-
E of this dissertation. 2-Aminoethyisulfuric (Fig. 5)
was a crystalline compound supplied by Dr. J. V. Karabinos. Sodium chondroitinsulfate (Fig. 6) was prepared
by this worker as described in Sec. III-A of this disser-
tation. Standard sodium heparinate (Figs. 7-9) was
purified heparin supplied by Hoffman-La Roche, Basle,
Switzerland.

N-Deacetylated and sulfated sodium chondroitinsulfate
(Fig. 10) was prepared by this worker as described
in Sec. III-C-1 of this dissertation. N-Deacetylated
and sulfated chitin (Fig. 11) was prepared by Dr. T. M.
Shen Han of this laboratory. The two acetylated sodium
chondroitinsulfates (Figs. 12,13) were prepared by this
worker as described in Sec. III-D of this dissertation
and as discussed in Sec. IV-D (the sample for Fig. 13
being prepared by acetylation at 0°C.). Partially N-
deacetylated sodium chondroitinsulfate (Fig. 14) was
prepared by this worker by the strong alkali procedure
as described in Sec. III-B-5 of this dissertation.
Partially N-deacetylated sodium chondroitinsulfate
(Fig. 15) was prepared by this worker by the amide ex-
change procedure as described in Sec. III-B-6 of this
dissertation. The infrared spectrum of water was ob-
tained by placing a few drops of distilled water on a
badly damaged (and otherwise useless) sodium chloride
cell followed by exposure in the infrared machine.
This worker would like to express his appreciation to
Mr. Stanley L. Kopczynski of The Ohio State
University, for his aid in obtaining many of these in-
frared spectra.
FIGURE 2
SULFAMIC ACID
REFERENCE STANDARD
SAMUEL P. SADLER & SONS INC.

FIGURE 3
SULFAMIC ACID
FIGURE 10
SULFATED SODIUM CHONDROITIN
SULFATE

FIGURE 11
SULFATED CHITOSAN
FIGURE 12
ACETYLATED SODIUM CHONDROITIN SULFATE I

FIGURE 13
ACETYLATED SODIUM CHONDROITIN SULFATE II
FIGURE 14
PARTIALLY N-DEACETYLATED SODIUM CHONDROITIN SULFATE I

FIGURE 15
PARTIALLY N-DEACETYLATED SODIUM CHONDROITIN SULFATE II
PERCENT TRANSMITTANCE

WAVE NUMBERS IN CM⁻¹

1000 900 800

2000 1500 1000

3000 2500 1500

4000 3000 2500

WAVE LENGTH IN MICRONS

FIGURE 16

WATER
V. SUGGESTIONS FOR FURTHER RESEARCH

1. Completely N-deacetylated and relatively undegraded chondroitin-sulfuric acid should be prepared by the improvement of present methods or by the development of new methods.

2. Completely N-deacetylated chondroitin-sulfuric acid should be sulfated by methods now developed and the product evaluated with regard to anticoagulant activity, electrophoretic behavior and chemical properties.

3. Completely N-deacetylated chondroitin-sulfuric acid should be characterized as to structure. This should be approached by means of periodate studies and the preparation of acetylation and methylation derivatives followed by hydrolysis and identification of the products.

4. Chondroitin-sulfuric acid should be tosylated in formamide solvent and the rate of tosylation compared with that of completely N-deacetylated chondroitin-sulfuric acid.

5. Chondroitin-sulfuric acid should, if possible, be selectively desulfated and the product subjected to periodate and tosylation studies.

6. Hydrolysis studies, in acid medium, should be
conducted on ethanolamine disulfate so as to gain more insight into the relative stabilities of O-sulfate and N-sulfate.

7. The two possible monoacetyl derivatives of ethanolamine should be prepared and sulfated. These monosulfation products should be characterized.

8. Attempts should be made to desulfate acetylated chondroitinsulfuric acid. The product should be characterized with regard to periodate consumption and tosylation rates.

9. A very thorough study should be made of the infrared spectra of the available derivatives of chondroitinsulfuric acid as well as heparin, ethanolamine-disulfate, sulfated chitin, sulfated glucosamine and derivatives thereof. This should lead to a very definite allocation of the absorption region of the N-sulfate group.

10. Sulfated chondroitinsulfate should be characterized with regard to molecular weight by means of light scattering.

11. Various molecular weight sulfated chondroitinsulfate samples should be prepared and analyzed with respect to anticoagulant activity and toxicity.

12. Acetylated chondroitinsulfuric acid should be selectively desulfated and the product converted to other derivatives.
13. Attempts should be made to isolate the disaccharide of chondroitinsulfate which would be produced by hydrolysis of the other glycosidic linkage than the one hydrolyzed in the isolation of chondro- sine. Possibly this desired compound could be isolated from an acid hydrolysis of a chondroitinsulfate derivative. This new disaccharide should be characterized.

Also, attempts should be made to control the acid hydrolysis in such a manner that the reaction could be stopped when a maximum amount of tetrasaccharide was present in the solution. This compound should then be isolated, possibly by chromatography, and characterized.
VI. SUMMARY

1. A procedure has been developed for the purification of sodium chondroitinsulfate from crude commercially available sodium chondroitinsulfate. It has been shown that this chromatographic procedure yields a product which is chemically pure and exists as a stable hydrate under specified drying conditions. Further, it has been shown that protein contamination of the crude starting material may be effectively removed without resorting to glacial acetic acid precipitation. This neutral sodium salt has been converted to the sodium acid salt and the calcium salt and these latter materials characterized.

2. Sodium chondroitinsulfate has been partially N-deacetylated by means of concentrated sodium hydroxide to yield a substantially undegraded product which has been shown to have an acetyl content of approximately 0.6 groups per disaccharide unit. Preliminary studies employing amide exchange (or amine acylation) have produced a partially N-deacetylated sodium chondroitinsulfate, in good yield, analysis of which has indicated an acetyl content of 0.24 acetyl groups per disaccharide unit.

3. Partially N-deacetylated sodium chondroitinsulfate, prepared by the strong alkali method, has been
sulfated in an activated state by means of chlorosulfonic acid and pyridine to yield a nondialyzable product, in good yield, containing approximately 3.5 sulfate groups per disaccharide repeating unit.

Partially N-deacetylated sodium chondroitinsulfate, prepared by the strong alkali method, has been homogeneously sulfated by means of chlorosulfonic acid and pyridine in formamide solvent to yield a product which contains approximately 3.4 sulfate groups per disaccharide repeating unit. The products obtained by each procedure have been characterized chemically.

It has been found that the sulfation of partially N-deacetylated sodium chondroitinsulfate by means of pyridine-chlorosulfonic acid leads to a product with approximately 40 per cent of the in vitro anticoagulant activity of standard sodium heparinate and approximately the same in vivo toxicity of the latter material. Clinical studies have shown that this sulfated chondroitinsulfate did not prevent the clotting of rabbit and dog blood, in vitro, as long as standard sodium heparinate.

4. Sodium chondroitinsulfate has been homogeneously acetylated by means of acetic anhydride and pyridine in formamide solvent to yield a non-dialyzable product with an acetyl content such that the presence of 4.0-4.2 acetate groups per disaccharide repeating
unit was indicated. None of the sulfate present in the starting material was removed during acetylation. This product has been characterized.

5. The crystalline disodium salt of 2-sulfamino-ethylsulfuric acid trihydrate has been prepared by the sulfation of ethanolamine with sulfur trioxide and pyridine. This product, obtained in good yield, has been characterized chemically.

6. An infrared spectrographic study has been made of the derivatives of chondroitinsulfuric acid prepared by this worker, heparin, sulfamic acid, sulfated chitin, 2-sulfaminoethylsulfuric acid trihydrate (sodium salt). From this study the location of the \( N \)-sulfate group absorption band has been tentatively designated as occurring at 1,000 cm\(^{-1} \) in the infrared spectra of these materials.

7. Evidence has been presented indicating that the anticoagulant activity of \( N \)-deacetylated and sulfated chondroitinsulfuric acid is proportional to the degree of \( N \)-deacetylation of this material prior to sulfation. As a result, the anticoagulant activity of the final sulfated product has been shown to be related to the number of \( N \)-sulfate groups occurring per disaccharide unit.
VII. BIBLIOGRAPHY


(82) S. Bergström, Naturwissenschaften, 22, 706 (1935); Hoppe Seyler's Z. physiol. Chem., 238, 163 (1936).


(5) A. F. Charles and D. A. Scott, J. Biol. Chem., 102, 431 (1933).


K. Freudenberg and M. Harder, Ann., 433, 230 (1923).


A. Gronwall, B. Ingelmann and H. Mosimann, Upsala Lakorfören Förh., 50, 397 (1945); Chimie & Industrie, 55, 206 (1946).


J. Hebting, Biochem. Z., 62, 353 (1914).


(21) R. Kuhn and H. Roth, Ber., 66, 1274 (1933).


(40) T. Miyazaki, J. Biochem. (Japan), 20, 211, 223 (1934).


(48) J. Müller, Ann., 21, 277 (1837).


(115) P. Rathgeb, Research Report for the Period October 1, 1949 to September 30, 1950, Project 11670-165 This Laboratory.
(15) M. Reinert and A. Winterstein, Arch. intern. pharmacodynamie, 62, 47 (1939).
(98) C. R. Ricketts, Research, 6, 17s (1953).
(108) Miss T. M. Shen (Han), Ph.D. Dissertation, The Ohio State University (1954).


(86) I/S Solusol, Danish Patent 65269 (1946).


(85) W. Traube, B. Blazer and E. Ludeman, Ber., 65, 603 (1930).


(38) B. Winter, Biochem. Z., 246, 10 (1932).


(9) F. Zuckerkandl and L. Meissner-Kleberman, Biochem. Z., 236, 19 (1931).
VIII. AUTOBIOGRAPHY

I, Charles Gene Summers, was born, January 2, 1928, in Akron, Ohio. I attended public schools and high school in Charleston, W. Va. I attended Morris Harvey College and was graduated with a degree of Bachelor of Science (Chemistry and Mathematics) in 1949. After graduation I was employed by the American Viscose Corp., Fredericksburg, Va. as a chemist. At the end of one year I resigned to continue my studies. At this time I enrolled in the Graduate School of The Ohio State University, Columbus, Ohio. From October, 1950 to June, 1954 I held the position of Research Fellow at The Ohio State University. From 1950 to the present I have been completing the requirements for this degree of Doctor of Philosophy at this institution. I have been employed since December, 1954, as a research chemist by The Nestle Co., Inc., Marysville, Ohio and have been engaged in off-campus research from The Ohio State University during this time.