PREPARATION AND CHARACTERIZATION BY STREAMING BIREFRINGENCE OF SODIUM DESOXYRIBONUCLEATE

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ABSTRACT

Three preparations of the sodium salt of desoxyribonucleic acid were isolated from calf thymus by a method expected to give minimal or no depolymerization. In addition, preparations were made from calf spleen and bull testis tissues.

Streaming birefringence measurements indicate that the distributions of molecular length are identical in the calf thymus preparations. The apparent molecular lengths range from 7,500 to 11,500 Å. The sodium desoxyribonucleate from testis appeared to be significantly shorter; that from spleen was intermediate between thymus and testis. The distributions of molecular length of two calf thymus preparations isolated by N. Simmons were compared with those prepared here.

Several explanations are put forth to explain the polydispersity and discrepancies between preparations.

TABLE OF CONTENTS

PART I

INTRODUCTION

While working with pus cells in the laboratory of Hoppe-Seyler in 1868, Friedrich Miescher showed that the nuclei of these cells contained an unusual phosphorus compound, now recognized as nucleoprotein. Miescher, who may be regarded as the founder of the chemistry of the cell nucleus, isolated the acidic part of this phosphorus compound from salmon sperm in 1872 and showed that this acidic substance constituted almost 50% of the weight of the sperm. Since then, this compound, desoxyribonucleic acid, has been isolated from many tissues. It is only recently, however, with the advent of new cytological, biochemical, and polymer structure techniques, that the nature of nucleic acid is being revealed.

Nucleic acid consists of three main components: organic bases (purines and pyrimidines), a pentose sugar, and phosphate. Two main types of nucleic acid may be distinguished. One, ribonucleic acid (RNA), contains D-ribose as the sugar component and is found largely in the cytoplasm; the other, desoxyribonucleic acid (DNA), contains D-2-desoxyribose and is found almost exclusively in the nucleus. This study is concerned only with DNA. The basic components of DNA are the purine bases, adenine and guanine, and the pyrimidine bases, cytosine and thymine. Recently, a small amount of another pyrimidine, 5-methyl cytosine, has been found (1). The molar

proportions of the bases in thymus DNA, as determined by paper chromatography, are cytosine : adenine : guanine: thymine as 1.0 : 1.6 : 1.3 : 1.4 (2).

A purine or pyrimidine is condensed with a pentose or desoxypentose to form a nucleoside. In nucleosides, the sugar occurs in the furanose configuration (3) and is attached to the base by a N-β-D-glycosidic linkage (4), at N_3 in the case of the pyrimidines (5) and at N_9 in the case of the purines (6). The phosphoric esters of the nucleosides are called nucleotides, and may be obtained from DNA by enzymic hydrolysis (7). Carter, who isolated desoxyribosenucleotides by enzymic hydrolysis, has obtained evidence which shows that one phosphate is attached to C_5 in the sugar (8).

DNA is a high polymer composed of many nucleotides joined together by phosphate ester linkages. For this reason, it is more appropriate to term the compound desoxyribosepolynucleotide, and to specify the name of the animal and tissue from which it is prepared; for example, calf thymus desoxyribosepolynucleotide. However, for the sake of convenience and in conformity with common usage, the generic term desoxyribonucleic acid is retained.

The biological properties of DNA are dependent upon its high degree of polymerization which has been confirmed by various physical chemical studies (9). Even the Feulgen reaction (10), the common method of identification of DNA in tissues, works only upon the polymer, since it is contingent upon the insolubility of the high polymer in tissues (11).

DNA functions as a transforming factor or mutating agent (page 4) only when it is in the undegraded state.

One of the primary problems is to confirm the internucleotide linkage in DNA. Since C_4 in the sugar is occupied in ring formation and C_2 carries no hydroxyl group, only the hydroxyl groups in positions 3 and 5 are available for internucleotide linkages. On the basis of titration data and enzymic evidence, it is believed that the mode of linkage is C_3-C_5 (9); i.e., C_3-phosphate residue-C_5.

A recently proposed structure for DNA is that of Watson and Crick, (12) who propose a structure of two right-handed helical chains, each coiled about the same axis. Each chain consists of phosphate diester groups joining β-D-desoxyribofuranose residues by means of C_3-C_5 linkages. The bases are on the inside of the helix with their planes perpendicular to the fibre axis, and the phosphates are on the outside where they are easily accessible to cations. The chains are held together by hydrogen bonding between purine and pyrimidine bases.

The structure of Watson and Crick is primarily concerned with relative spatial positions of the bases, sugar, and phosphate. Such a structure leaves the problem of macromolecular configuration unanswered. Since the biological properties of DNA appear to be dependent upon the macromolecular configuration, it seems desirable to study this problem. The major obstacle to such a study is dependence of the macromolecular properties upon the method of preparation, since most prepara-

tions result in some depolymerization. However, if a method of preparation is utilized which does not cause depolymerization of the molecule, a study of macromolecular configuration may provide information which would shed light on the biological properties.

Determinations of the DNA content of various tissues are found to be in good accord with the chromosome theory of heredity. Mirsky and Ris (13) showed that, in a given organism, the same quantity of DNA per nucleus is present in all tissues which have two sets of chromosomes per nucleus. The sperm nucleus, which contains only one set of chromosomes, contains one-half as much DNA per cell.

In the nucleus, DNA is complexed with the basic proteins, histone or protamine, to form nucleoprotein. Protamine, a simple protein whose primary constituent is arginine (14), has been found only in sperm nuclei, while histone, a larger and more complicated protein, has been found in most other tissues (14). In addition to protamine and histone, a complex acidic protein has been isolated from preparations of pure chromosomes (14). According to Davidson's interpretation (15), this tryptophan-containing protein is the essential basis of the chromosome thread and is embedded in a nucleic acid or nucleohistone matrix. Mirsky, on the other hand, believes that the morphological configuration of chromosomes is dependent upon the combination of DNA and residual protein; DNA is part of the germinal material and histone does not contribute to the structure of the chromosome (14).

Another reason for postulating DNA as part of the germinal

material is the ability of certain DNA to transform one type of bacterial cell into another with different morphological characteristics. In 1944, Avery, McLeod, and McCarty (16) isolated from encapsulated pneumococcus cells of Type III a biologically active fraction, which, under appropriate cultural conditions, was able to induce the transformation of unencapsulated Type II pneumococcus into fully encapsulated Type III pneumococcus. This transforming principle was subsequently proven to be pure DNA (17). The power of this DNA as a transforming agent is destroyed upon depolymerization (18).

The objectives of the present study are:

1. To prepare the sodium salt of calf thymus DNA by a method which causes little or no depolymerization and to carry out multiple preparations to test its reproducibility.
2. To apply this method to preparation of the sodium salt of DNA from calf spleen and bull testis.
3. To compare these preparations with regard to molecular length.

PART II

PREPARATION OF SODIUM DESOXYRIBONUCLEATE

History.

In the isolation of DNA from fish sperm, Miescher extracted the cytoplasm with dilute acid and then separated the nucleic acid from the basic protein, protamine, with dilute alkali. Miescher warned, however, against prolonged contact with alkali or acid (19). Subsequent investigators did not heed this warning. The early investigators of nucleic acid were interested only in obtaining a product which they could analyze chemically. When evidence for the existence of polynucleotides of higher order was uncovered (20), interest in preparing undepolymerized DNA arose. In 1924, Hammersten and Ban (21) developed a method for preparing a highly polymerized sodium desoxyribonucleate (SDN) from thymus. (Thymus was the animal tissue usually used because its content of nuclei per mass of tissue is relatively large.) They extracted the nucleoprotein complex with distilled water, precipitated it with saturated $CaCl_2$, dissolved the precipitate in 10% NaCl, and then dissociated the complex in saturated NaCl. The protein was filtered off and the nucleic acid was precipitated with alcohol. The yield of approximately 2% was slightly larger than that of most present day preparations. Although Hammersten worked at 0° C. with solutions that were neutral to litmus, some depolymerization did result in this preparation (22), due, perhaps, to lack of

provisions for inhibition of depolymerizing enzymes.

When the relation between the biological properties and the degree of polymerization of DNA was realized, new attempts to prepare an undegraded product were made. Up to the present time, the most satisfactory method for isolating thymus nucleoprotein is that of Mirsky and Pollister (23), which makes use of solubility differences of nucleoprotein in NaCl solutions of various concentrations. The tissue is thoroughly extracted with physiological saline at 0° C. to remove cytoplasmic material. The remaining nuclear material is dispersed in 2 M NaCl and the nucleoprotein is precipitated from the viscous solution by reducing the NaCl concentration to 0.14 M. Mirsky and Pollister isolated nucleic acid from this nucleoprotein preparation by shaking the nucleoprotein with a chloroform-octyl alcohol mixture according to the procedure of Sevag (24). Although the method may be applied to many tissues, Marko and Butler (22) stated that the yield is poor even with thymus and that the method is quite time consuming.

Gulland, Jordan and Threlfall (25) applied to calf thymus the Mirsky technique of isolating the nucleoprotein, and decomposed the nucleoprotein by the method of Sevag to obtain a product which was, perhaps, the most highly polymerized preparation obtained up to that time. However, Doty (26) showed by a light scattering study that the Gulland preparation gave a significantly lower molecular weight for calf thymus SDN than did the preparation by the Schwander and Signer method (27). In the preparation of Schwander and Signer, the nucleoprotein was extracted at 0° C. with 1 M NaCl containing 0.01 M

sodium citrate as an enzyme inhibitor. (All solutions used in the preparation contained 0.01 M sodium citrate.) The nucleoprotein was precipitated in 0.14 M NaCl and then redissolved and reprecipitated twice more. This part of the procedure closely followed Mirsky's isolation of nucleoprotein. The novel features were the dissociation of the nucleoprotein in saturated NaCl and the removal of the protein by filtration through Celite (diatomaceous earth) which was faster and more effective than centrifuging. The SDN was precipitated in alcohol and the washed, dried product had the appearance of white asbestos fibre. The primary disadvantage was that the preparation took approximately one month to carry out.

An improved method of preparation of calf thymus SDN involving the use of a detergent, sodium dodecyl sulfate, was announced almost simultaneously by two groups of investigators working independently, Marko and Butler (22) and Simmons, Kay, and Dounce (28). In both procedures, the nucleoprotein was extracted by the Mirsky method. The Simmons procedure called for use of a 5% solution of sodium dodecyl sulfate to dissociate the nucleic acid from the protein in 1 M NaCl. The precipitated protein was removed by centrifugation and the nucleic acid was precipitated from the supernatant liquid with alcohol. The yield was very high (3% of the tissue weight). The Marko and Butler technique involved the determination of nitrogen concentration to fix the N at 0.5 mg. per ml. and the sodium dodecyl sulfate at 0.5% in 1 M NaCl. The precipitate was

filtered off through Celite. Although the yield obtained was not as high as that of the Simmons group, the product appeared purer and the procedure was successfully applied to other tissues such as calf spleen, bull testis, and fowl erythrocyte.

Criteria Followed in Simmons Preparation. The following preparation was adapted from a method recently developed by N. S. Simmons (29). It conforms to certain criteria which are necessary for giving a highly polymerized preparation:

1. Enzymatic degradation is minimized by collecting the tissue immediately after death of the animal and storing at -60° C. in dry ice (27).
2. There is no rise above 5°C. in the preparation, prior to adding an agent which will destroy any enzyme which may be present (29). After this, work may be carried out at room temperature.
3. There is no variation from pH 7.
4. No reagents are used which will degrade, complex, or oxidize the DNA.
5. The SDN can be easily isolated within two days, a large improvement over other preparations.
6. No techniques are employed which may result in degradation.

Procedure. Calf thymus is collected within 15 minutes after slaughtering, quickly frozen, and stored under solid CO_2 until needed. Then it is thawed, cleaned, and minced by hand with a knife.

Starting material: 200 g. of cleaned, minced thymus.

1. All operations at 0°C. Homogenize the gland in the blendor 15 minutes with 100 ml. 0.15 M NaCl-0.01 M sodium citrate. Dilute to 600 ml.

2. Centrifuge at 0° C, 800 x g for 20 minutes. Discard the supernatant liquid.

3. Homogenize the sediment in the blendor 5 minutes with 75-125 ml. of the salt solution and centrifuge as above. Repeat this procedure 3 or 4 times until the supernatant liquid is clear.

4. Dissolve sediment in 30% sodium xylene sulfonate, total volume 1 liter. Then work at room temperature. Stir for 1 hour.

5. Add 2 volumes salt solution. Stir $\frac{1}{2}$ hour.

6. Filter off precipitate through coarse Celite two times or until filtrate is just slightly turbid.

7. Filter with Hyflo Super-Cell two times or until clear.

8. Add about 0.40 volumes 98% isopropyl alcohol.

9. Dissolve precipitate of SDN in 900 ml. water, add 100 ml. 3 M sodium acetate (pH 7.0) and precipitate with 530 ml. 98% isopropyl alcohol. Repeat the dissolution and precipitation 2-4 additional times, filtering the aqueous solution before the last precipitation with alcohol.

10. Wash the precipitate 3 times with 95% ethyl alcohol, 5 times with dry acetone, and twice with dry ether.

Yield: approximately 2 g.

Detailed Discussion of Preparation. The following preparatory method applies in detail only to calf thymus gland. The necessary modifications for calf spleen and bull testis will be stated afterwards. All quantities are given on the basis of a starting material of 200 g., wet tissue weight.

1. Isolation of the Nuclei

It is important that the glandular material be collected as soon as possible after the slaughtering of the animal (preferably within 15 minutes), since the nucleoproteins undergo autolytic degradation (23) after the animal has been killed. The glands are quick-frozen in dry ice and kept at -60° C. under dry ice until the preparation is begun.[1] All operations are carried out at 0° to 5° C. The gland is thawed, the sheath of connective tissue is removed with a knife, and the glandular material is chopped into small pieces. For convenience, it is divided into two equal portions. One portion is put into a 1 l. Waring blendor cup. One hundred ml. of a salt solution which is 0.15 M in sodium chloride and 0.01 M in sodium citrate[2] and 5 to 8 drops of n-octyl alcohol,[3] which suppresses foam (22), are added. The mixture is minced in the

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[1] It has been found that nucleoproteins undergo autolytic degradation in tissues in cold storage at -40° C. (31).

[2] The use of sodium citrate is based upon the observation that pancreatic desoxyribonuclease requires magnesium ion which is complexed by citrate, thereby inhibiting the enzyme (32). However, recent experiments (33) indicate that cellular desoxyribonuclease is a different enzyme from the pancreatic enzyme, and the value of the citrate as an inhibitor for the cellular enzyms has been questioned (28). Nevertheless, the use of citrate has been continued.

[3] The presence of n-octyl alcohol in the initial dispersal of minced tissue in salt solution causes hemolysis and facilitates the removal of hemoglobin (34), but the tissues have to be

blendor for 15 minutes. The blendor is connected to a variac so that its speed can be controlled. The speed is set just fast enough so that it will cut open the cells. Running the blendor at full speed results in high shear gradients which are believed to degrade the DNA (28); also at high speeds, air is drawn into the solution causing undesirable surface effects (29).

Four hundred ml. of the salt solution are added to the hemoglobin-colored mixture in the blendor, and the mixture is beaten for 1 to 2 minutes until homogeneous. Then it is centrifuged at 800g[4] (2000 R.P.M. on the International PR-1 Refrigerated Centrifuge) at 0° C. for 20 minutes.

The red supernatant liquid is decanted. The precipitate of unminced glandular material, nuclei, and connective tissue is taken up with the salt solution and placed in the metal semi-micro head of the Waring blendor. A total of 100 ml. of the salt solution is added, and it is stirred for 5 minutes. The mixture is poured into the 1 l. blendor cup, 400 ml. of salt solution added, and the mixture is homogenized for 1 to 2 minutes.[5] It is again centrifuged for 20 minutes at 800g and 0° C.

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washed a great deal to remove the color of hemoglobin.

[4] This speed is known to sediment nuclei, chromatin and unminced fragments while leaving the cytoplasmic constituents in the supernatant liquids (29).

[5] Long strands of white connective tissue often wrap around the blendor blades. In decanting, these will remain attached to the blades, providing an efficient means of separating this connective tissue, which can usually be discarded. However, in the initial stages of the extraction, the connective tissue often occludes glandular material which can be detected by a

The connective tissue, which remains wrapped around the blendor blades, is discarded.

The second portion of thymus gland is treated in the same manner. After the second centrifuging, the sediments of both portions are small enough so that they may be combined. They are then taken up in the salt solution, poured into the metal Waring cup, and a total of 100 to 150 ml. of salt solution (depending on the size of the precipitate) is added, and the pale yellow mixture is beaten for 5 minutes. It is poured into the 1 l. Waring cup, 400 ml. of the salt solution added, and the mixture homogenized for 1 to 2 minutes. It is again centrifuged for 20 minutes at 800g and 0° C.

This entire process beginning with the homogenization in the metal cup is repeated 2 or 3 times until the supernatant liquid is colorless and clear. After the last mixing of the combined precipitates in the blendor, the blendor blades should be free of connective tissue.

2. Separation of the Sodium Desoxyribonucleate from Protein

The white sediment of chromatin threads contaminated with some fibres (28) is taken up in 100 ml. of the salt solution and poured into the 1 l. blendor cup. A solution of 300 g. of sodium xylene sulfonate[6] in 600 to 700 ml. of distilled water

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reddish coloring. In this case, the connective and glandular tissue are beaten with a small amount of the salt solution, the liquid decanted and added to the rest of the mixture of nuclei and the salt solution.

[6] The sodium xylene sulfonate is obtained from Wyandotte Chemical Corporation as a commercial detergent, Naxonate. Naxonate is made by sulfonating industrial grade xylene

is poured slowly into the blendor with rapid stirring. The light brown mixture becomes extremely viscous, with the chromatin remaining in a large white viscous mass on the surface of the mixture until the blendor's blades gradually suck the chromatin down and disperse it. After 5 minutes the mixture is homogeneous and highly viscous. The mixture is stirred in the blendor for an additional 5 minutes using the precautions cited above.

The mixture is made up to 1 l. with the salt solution and mixed in a 4 l. beaker with vigorous stirring for 1 hour at room temperature.[7] Some air is stirred into the solution and the color becomes lighter. Two liters of the salt solution are added and the resulting light brown mixture is stirred for $\frac{1}{2}$ hour. A precipitate of protein can be detected.

The function of the detergent, sodium xylene sulfonate, is to coat the protein and separate it from the nucleic acid. The proteins present in nucleoproteins are the basic histones which have an isoelectric point of pH 12. At pH 7 during the preparation, the ionization of the carboxyls is suppressed, and the histones are positively charged. The negative xylene sulfonate ions, which have a high binding strength, are firmly attached to the NH_3^+ groups of the protein. In excess of the detergent, another layer of xylene sulfonate ions is absorbed

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which gives a mixture of the various sulfonates. For use in this preparation it is recrystallized from 95% ethanol (300 g. Naxonate in 1 l. of alcohol) with approximately 50% recovery.

[7] All subsequent operations are carried out at room temperature.

with its aromatic group probably adjacent to the aromatic group of the inner layer and its negative sulfonic group on the outside (35). This gives rise to a dispersing effect resulting in a solubilizing of the complex. When the concentration of the surface active agent is reduced by dilution, the outer layer is removed. The remaining layer of detergent just neutralizes the electric charge on the protein, causing a loss in its residual water of hydration, with consequent loss in stability resulting in precipitation of the protein-surface active agent complex.

A pad of coarse Celite[8] (either Celite 545 or 535) ¼ to ½ inch thick is laid down on a large (18½ cm.) Buechner funnel. It is important that a very fast, high wet strength filter paper be used (Schleicher and Schuld #410 was found to be the most satisfactory for the filtration).

The 30% sodium xylene sulfonate solution is carefully poured onto the Celite pad so that the Celite will not be dispersed. The first filtrate will be slightly turbid. A new pad of coarse Celite is laid down, and the solution is filtered a second time. If the filtrate is not almost clear, it is filtered again through the same pad of Celite. Then a 100 ml. beaker of Hyflo Super-Cell (a diatomaceous earth filter aid) is mixed into the solution and the resulting suspension is filtered through the same (second) Celite pad. Another

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[8] The coarse Celite contains many finer particles which pass through the filter paper if not removed first. This is done by mixing the Celite with water, allowing the suspension to settle for 20 minutes, and decanting the liquid which contains the suspended finer particles. This is repeated 3 or 4 times until the liquid is clear after standing for 20 minutes.

beaker of Hyflo Super-Cell is added and the filtration is repeated giving a crystal-clear colorless filtrate.[9]

3. Precipitation and Purification of the Sodium Desoxyribonucleate

A volume of 98% isopropyl alcohol (Eastman reagent grade) that is 0.45 the volume of the clear filtrate is stirred into the clear filtrate. The SDN precipitates in the form of long white fibres which coil around the stirring rod and which may be lifted out. The filtrate should be tested for additional precipitate with 0.05 volume (of the filtrate) portions of isopropyl alcohol. In many cases, the first 0.05 volume portion of alcohol yields additional precipitate.

The precipitate is placed in the large Waring cup and dissolved in 500 to 600 ml. of distilled water. It is stirred as gently as the blendor will permit, care being taken not to beat any air into the solution. The solution is then made up to 900 ml., mixed, and 100 ml. of 3 M sodium acetate buffer[10] (pH 7.0) is stirred into the solution. Then 530 ml. of isopropyl alcohol are added with a great deal of stirring. The SDN is again taken up on a stirring rod, and the solution is tested for completeness of precipitation with 10 to 20 ml. portions of alcohol. This reprecipitation is repeated 2 to 3 times. Before the last reprecipitation, the water solution of

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[9] Occasionally a light yellow color is present due to an impurity in the Naxonate; also the Hyflo Super-Cell sometimes contains a light yellow coloring. This color is not present in the precipitate.

[10] The pH of the 3 M sodium acetate solution is adjusted with glacial acetic acid.

the SDN is filtered through a Buechner funnel to remove any debris which may be present. The filtrate is clear and colorless.

In order to obtain a final product in the form of small particles rather than long fibers, a somewhat different method of adding alcohol is employed for the last precipitation. The SDN solution is made up to 1 l. as before and placed in a large beaker. Five hundred and thirty ml. of isopropyl alcohol are placed in a graduated separatory funnel and slowly added into the vortex created by the mechanical stirrer. The stirring and addition are carried out slowly so that no long fibres of sodium desoxynucleate are formed. The last 50 ml. are added dropwise. Within a few ml. of the end point the stirring is slowed down. With further dropwise addition of isopropyl alcohol, the solution becomes turbid, and at the appearance of an opalescent sheen to the solution, alcohol addition and stirring are stopped. This end point is very sensitive. Addition of a few drops of alcohol beyond this point causes precipitation of the sodium desoxynucleate in the form of long fibres, and the process has to be repeated.

The colloidal solution is allowed to stand for 15 minutes. Then 10 to 20 ml. of isopropyl alcohol are added slowly with a small amount of stirring. A precipitate of small particles may begin to settle out after 15 minutes. If this is not observed, another 10 to 20 ml. portion of isopropyl alcohol is added. Within four such additions, larger particles will form and precipitate. The precipitate is allowed to settle overnight.

After the period of settling, the supernatant may be decanted leaving just enough of the liquid to cover the precipitate. Two hundred to three hundred ml. of 95% ethyl alcohol are added to dehydrate the precipitate,[11] and the mixture is centrifuged.

The precipitate is washed on a small Buechner funnel (without suction) three times with 95% ethyl alcohol, three times with dry acetone, and twice with dry ether. The ether is allowed to evaporate in the air. The hygroscopic product of small white amorphous particles is put into a dessicator over $CaSO_4$.

The yield is approximately 2 g.

4. Modifications for Preparations from Calf Spleen and Bull Testis

Calf Spleen. Two hundred g. of gland are used as the starting material. The number of nuclei is considerably less than for the same starting weight of thymus. The spleen nuclei are also obscured by large amounts of connective tissue. This connective tissue is broken up into small pieces and does not wind itself around the blendor blades.

After the second centrifuging, the precipitates are taken up in the salt solution and passed through a wire mesh (20 mesh per inch is convenient) to remove the connective tissue. The preparation may then be treated in the same manner as the thymus. However, the amount of water in which the spleen SDN

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[11] This prevents the clumping of the precipitate into larger particles during centrifugation.

is dissolved should be reduced to 675 ml. since the final yield is much smaller than that obtained from thymus gland.

To obtain maximum recovery, the alcohol solutions are centrifuged.

Bull Testis. The product obtained from 200 g. of testicular tissue is very small. Therefore, additional precautions to insure maximum recovery are taken. The 200 g. of tissue need not be divided into two parts since the testis are easily minced and homogenized in the blendor. So many small pieces of connective tissue are present that it is very inconvenient to remove them with a wire mesh. Instead they are removed in the filtration, although this causes the filtration to be quite slow.

All precipitates from alcohol are centrifuged to obtain maximum recovery, and the precipitate is dissolved in only 450 ml. of distilled water.

PART III

DETERMINATION OF YIELD, CHEMICAL COMPOSITION, EXTINCTION COEFFICIENT, AND VISCOSITY

Yield.

In these preparations, no attempt was made to obtain large yields since this is not compatible with obtaining an undepolymerized product. In Table I are listed the starting wet weights (column 2) and the yield of SDN per 100 g. tissue (column 4). This can be compared with the results obtained in studies which attempted to estimate the total amount of DNA in various tissues.

Table I

1 Preparation	2 Wet weight (g.) of tissue	3 g. SDN prepared	4 g. SDN per 100 g. tissue	5 Total g. SDN per 100 g. tissue	6 Reference
Calf Thymus-1	125	0.92	0.74	2.5	(36)
Calf Thymus-2	100	0.49	0.49	2.5	(36)
Calf Thymus-3	232	2.0	0.86	2.5	(36)
Calf Spleen-4	220	0.32	0.15	(1.3)*	(37)
Bull Testis-5	290	0.082	0.028	--	--

*Determination obtained on rat spleen

The total amounts of SDN obtainable from the various tissues (column 5) are based on Davidson's determination (36) for calf thymus and Schneider's determination (37) for rat spleen since rat and calf tissues have similar amounts of DNA. However,

it should be pointed out that there is no general agreement among different workers in the field regarding the amount of DNA found in tissues. The low yield of SDN from bull testis as shown in Table I is of the same order of magnitude as that obtained by Marko and Butler (22).

Table II

Chemical Composition of SDN from Calf Thymus Preparation 1.

	Composition in per cent		
	Analytical determination	Statistical tetranucleotide*	Chargaff ratio of bases**
N	14.23	15.9	16.15
P	8.66	9.37	9.32
H	4.56	3.43	3.42
N:P	1.65	1.69	1.73

* Statistical tetranucleotide is based on equimolar ratio of adenine, guanine, cytosine, and thymine.
** Chargaff ratio of bases is listed on p. 2.

Chemical Composition of Product. Extensive analytical work has been carried out by Simmons (29) on the composition of calf thymus SDN obtained by the same preparative methods as employed here. His determinations agree with the results obtained by Chargaff (2) utilizing chromatographic techniques.

In view of this, and because chemical composition is not a useful tool in determining macro-molecular properties, only one analysis of chemical composition was carried out to check the possibility of protein contamination. In the past, investigators who prepared SDN used the nitrogen to phosphorus ratio as a criterion for determining whether any protein contaminant is present.

The results of this analysis of calf thymus SDN[12] (see Table II) are quite similar to the results obtained by Steiner (38) for a hydrated preparation made by the Gulland method. The nitrogen to phosphorus ratio of 1.65 indicates that no protein contaminant is present. The low values for both phosphorus and nitrogen are due to hydration. The discrepancy between the percent of hydrogen found in the determination and that in a statistical tetranucleotide corresponds to a hydration of 10.4% which agrees exactly with the hydration calculated from the nitrogen content. However, this does not agree with the hydration calculated from the phosphorus content (7.5%).

It is interesting to note that if the percentage of phosphorus in the sample is recalculated on the basis of the statistical tetranucleotide composition with a hydration of 10.4%, this yields a nitrogen to phosphorus ratio of 1.69.

SDN is very hygroscopic and its hydration changes markedly with slight changes in humidity. In order to obtain a reproducible extinction coefficient based on a weighed amount of SDN, it was necessary to hold the hydration constant. If all of the water is removed from SDN by means of P_2O_5 in vacuo at temperatures of 50° to 110° C., the macromolecular properties of the molecule undergo a marked change due to degradation (29). Anhydrous calcium sulfate at room temperature was therefore chosen as the standard state since this provides a

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[12] The analysis was carried out in the microanalytical laboratory of Kerchoff Laboratory, California Institute of Technology.

constant water vapor pressure and does not cause degradation due to dehydration.

The SDN sample used in the analytical work was taken from its standard state and placed over P_2O_5 at room temperature prior to analysis. The amount of water lost was 3.75% of the weight of the sample. This figure together with the hydration of 10.4% in the analyzed sample corresponds to a hydration of 13.7% in the standard state.

Determination of the Extinction Coefficient. Nucleic acid has a characteristic maximum in its absorption spectrum in the ultraviolet, which is attributed to the purine and pyrimidine bases. This absorption maximum at approximately 260mμ serves as a convenient means of determining the concentration of aqueous solutions. In order to determine an extinction coefficient, a definite amount of SDN in a constant state of hydration was weighed out. As stated above, the standard state was chosen as the weight over anhydrous calcium sulfate.

Table III

Weight (g.) of SDN Prep. 1	$E_{1\%}^{1\,cm.}$ at 260 mμ
0.113	211
0.0123	218
0.0100	174
0.0103	190
0.0132	194
0.0113	199
0.0962	207

All readings were carried out in a Beckman DU Spectrophotometer using a hydrogen lamp as the ultraviolet source and a slit width of 0.6 mm. Initially weighed amounts of SDN were

diluted with distilled water to give a concentration of approximately 0.0015% which could be conveniently read in the spectrophotometer. Table III shows that the extinction coefficient obtained by this method is not constant. Since it is known that the absorption of SDN is influenced by pH, experiments were undertaken to determine whether the pH of the solutions changed while being irradiated with ultra violet light in the spectrophotometer. It was found that the pH decreased roughly in proportion to the time the solutions were irradiated. In one experiment the decrease in pH was 1.5 units after 80 minutes of irradiation. This caused an increase of approximately 25% in the optical density. This was in accord with the findings of Oster and McLaren who noted a similar increase in absorption after irradiating a dilute solution of virus DNA (39). On the other hand, Frick (40) found that the maximum absorption at 260 mμ of SDN solution did not vary between pH3-8. In order to determine whether the change in pH was due to something in the distilled water itself, distilled water was irradiated in the spectrophotometer at 260 mμ. The same decrease in pH as that which took place in SDN solutions was noted. Subsequent experiments using conductivity water also gave the same results. This marked decrease in pH cannot be explained at this time.

In order to hold the pH constant, the optical density was determined in a KH_2PO_4-K_2HPO_4 buffer at pH 6.98 and ionic strength of 0.1. This gave constant results.

$E^{1cm.}_{1\%}$ at 260 mμ was found to be 170.4 and $E^{1cm.}$ for a solution containing 1 mole of phosphorus per liter is, therefore, 7030.

Table IV shows that the weight concentrations of various solutions agree well with concentrations determined from optical density measurements. The weights in Table IV were not as accurate as those used to determine the extinction coefficient in which 120 mg. of SDN were used. These values are presented only to show the close agreement between the weight concentration and the optical density concentration when the optical density is determined in a buffered solution.

Table IV

Preparation	Weight conc. % X 10^3	Conc. obtained by optical density % X 10^3
Thymus-1	22.2	21.4
Thymus-1	9.8	9.89
Thymus-2	9.2	9.20
Thymus-3	10.5	11.0
Spleen-4	10.3	10.5
Spleen-4	48.0	46.5
Testis-5	10.0	10.7
Testis-5	76.0	75.3

Figure 1 shows that the ultraviolet absorption spectra of SDN in buffer and in distilled water are slightly different. The peak at 260 mμ in distilled water is flattened out and slightly shifted in buffer. Nevertheless, the concentrations were determined by taking the optical density at 260 mμ in agreement with prevalent practice.

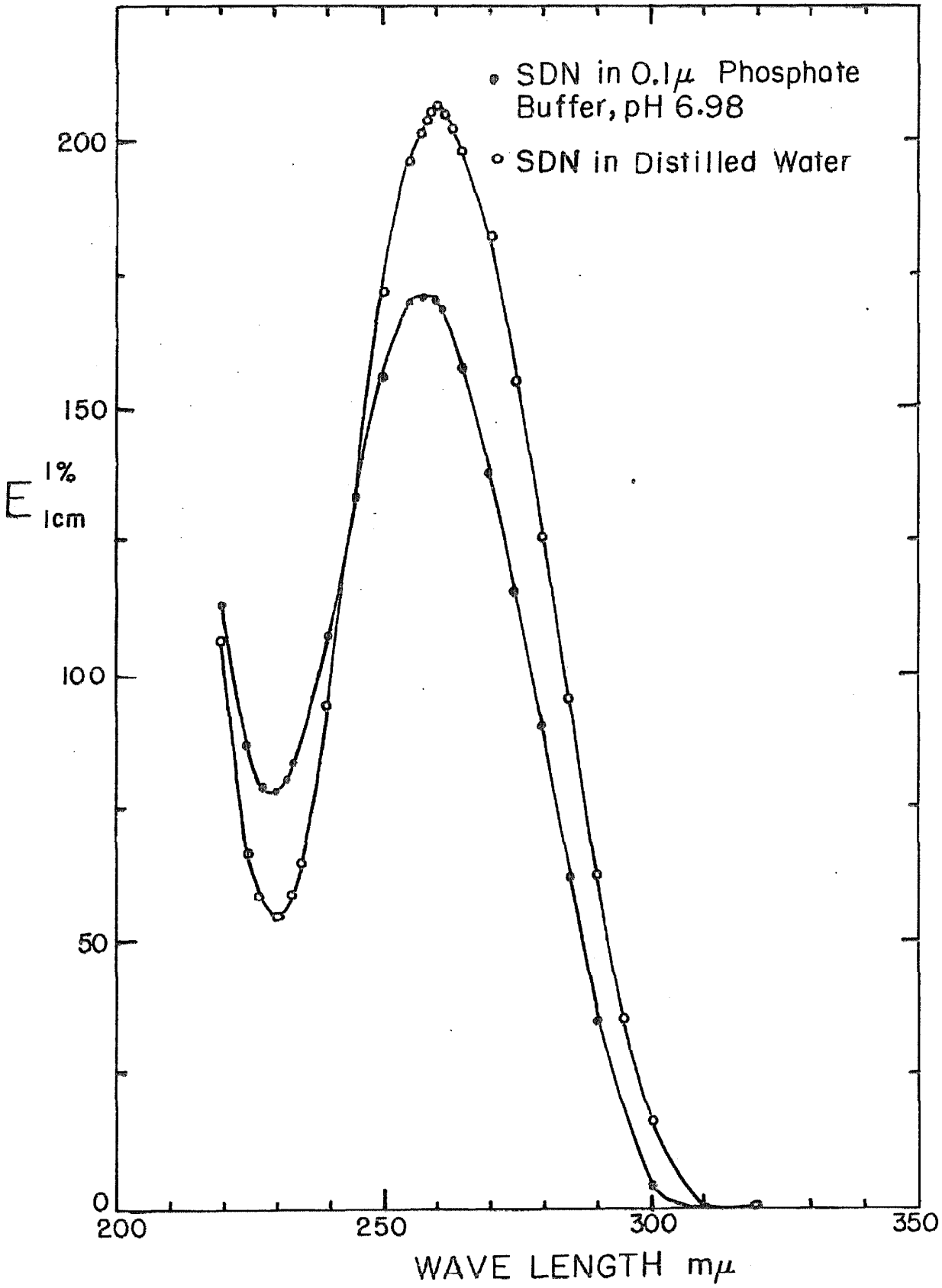

SDN in 0.1μ Phosphate Buffer, pH 6.98
SDN in Distilled Water
200
150
100
50
0
E 1% 1cm
200
250
300
350
WAVE LENGTH mμ

Figure I

It is difficult to compare the value for the extinction coefficient with that obtained by other investigators, since most determinations are made in distilled water or in buffers of unknown ionic strength and are based on samples whose hydration is not given.

Viscosity. The viscosity of dilute SDN solutions has been shown to be strongly dependent upon the gradient (41). This is caused by orientation of the asymmetric particles under the influence of a velocity gradient. The viscosity is also dependent upon pH at constant ionic strength as shown by Schwander (42). In the presence of even small amounts of salt the viscosity of SDN decreases markedly (43). Jordan has interpreted this behavior as indicating a coiling of the molecule caused by a reduction in the repulsion between the charged groups, thereby producing a more symmetrical molecule (44).

A set of viscosity measurements was made to investigate possible differences among the various preparations. The measurements were carried out in 0.15M NaCl with a K_2HPO_4-KH_2PO_4 buffer of ionic strength 0.1. An Ostwald capillary viscometer was used. The dimensions of the viscometer were: capillary length - 13.1 cm., capillary radius - 0.021 cm., volume - 5.0 $cm.^3$, pressure - 16 cm. of H_2O. The average gradient $\bar{G}$, for water, calculated from the equation

$$\bar{G} = \frac{8V}{3\pi r^3 t}$$

is 1860 $sec.^{-1}$ All measurements were carried out at 38.7° C.

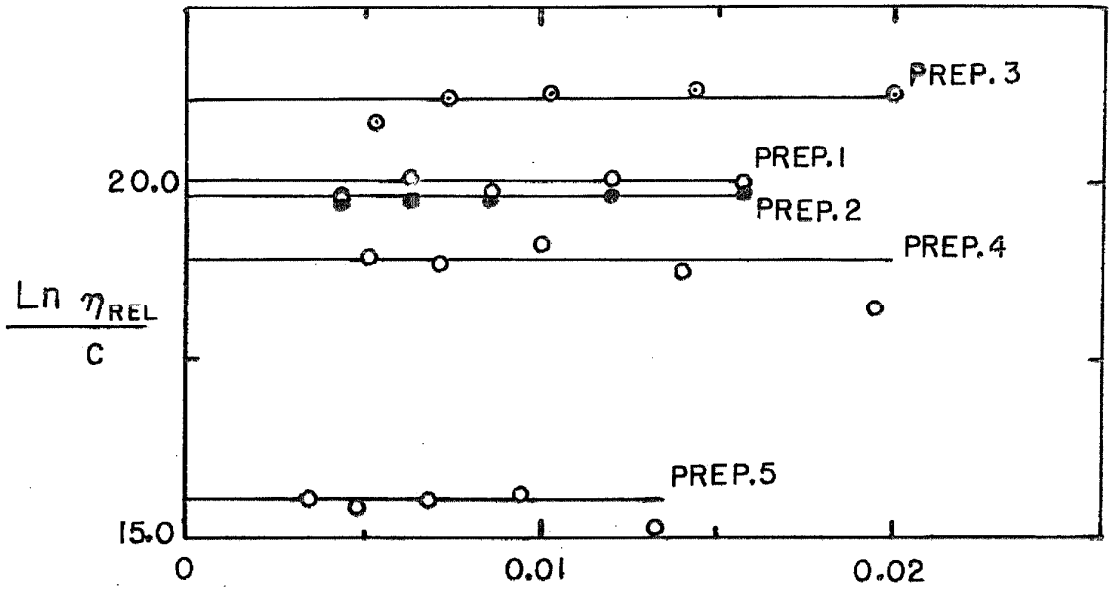
Table IV'

Prep.	Conc. (%)	t_{av} (sec)	t_o (sec)	$\frac{\frac{t_{av}}{t_o}-1}{c}$	$\frac{\text{Ln } \frac{t_{av}}{t_o}}{c}$
Thymus-1	0.0168	351.10	250.96	23.75	19.99
	0.0120	319.20		22.66	20.04
	0.00860	297.68		21.65	19.85
	0.00615	283.90		21.35	20.06
	0.00440	273.44		20.36	19.50
Thymus-2	0.0168	350.52		23.61	19.89
	0.0120	317.67		22.15	19.64
	0.00860	296.98		21.33	19.58
	0.00615	283.06		20.80	19.57
	0.00440	273.30		20.23	19.38
Thymus-3	0.0200	383.78		26.46	21.24
	0.0143	340.43		24.93	21.32
	0.0103	312.09		23.77	21.27
	0.00732	293.05		23.91	21.18
	0.00524	279.96		22.06	20.88
Spleen	0.0195	359.18		22.11	18.39
	0.0140	326.13		21.39	18.71
	0.0100	303.74		21.03	19.09
	0.00715	277.20		20.20	18.86
	0.00511	276.42		19.86	18.94
Testis	0.0133	308.67		16.77	15.14
	0.00950	291.05		16.81	15.60
	0.00680	278.83		16.34	15.49
	0.00486	270.41		15.95	15.36
	0.00347	264.86		15.97	15.54

Since only one viscometer was used, the intrinsic viscosities of the various preparations could be compared at a constant average gradient.

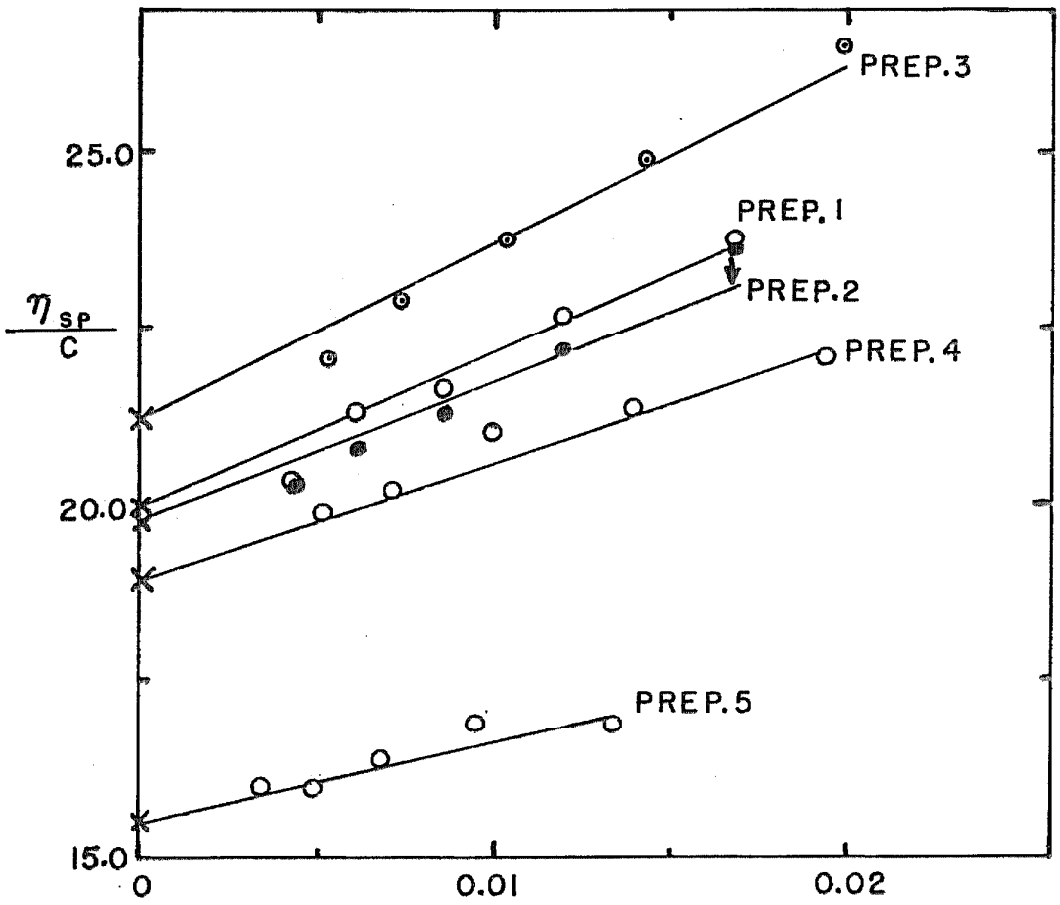
In table IV' are listed the average times of efflux (t_{av}) of various concentrations of the different preparations. This average time is a mean of 2 to 3 readings, all within 0.5 seconds of each other. t_o is a mean of 5 measurements. Ignoring the density (d) term, which is negligible in dilute solutions of nucleic acids, in the equation $\eta_{rel} = \frac{t_1 d_1}{t_2 d_2}$, the relative viscosity equals the ratio of the average time of efflux of the SDN solution to the time of efflux of the buffer. In figure 2 is plotted the natural logarithm of the relative viscosity divided by the concentration versus the concentration (in percent). In figure 3 is plotted the reduced specific viscosity (the specific viscosity divided by the concentration where $\eta_{sp} = \eta_{rel} = 1$ versus the concentration (in percent). The curves of $\frac{\text{Ln}\,\eta_{rel}}{c}$ versus concentration are horizontal lines, and are extrapolated to zero concentration. The limiting points can be plotted on the curves of $\frac{\eta_{sp}}{c}$ and used for extrapolation to zero concentration, since in the limit, $\frac{\text{Ln}\,\eta_{rel}}{c} = \frac{\eta_{sp}}{c}$.

The values of the intrinsic viscosity ($\lim_{c \to 0} \frac{\eta_{sp}}{c}$) indicate that small differences exist between preparations 1, 2, 3 and 4. Testis, however, has a much lower intrinsic viscosity than the other preparations. The estimated error in the time measurements is negligible when compared with the estimated error of 4% in the concentration measurements on these solutions. A 4%



CONCENTRATION OF SDN %

FIG. 2



CONCENTRATION OF SDN %

FIG. 3

error in concentration would make, in effect, the viscosities of preparations 1, 2, and 3 or 1, 2, and 4 identical. Thymus preparations 1, 2, 3 are shown by streaming birefringence to be identical in molecular length distribution. Streaming birefringence measurements also indicate that spleen is different from the thymus preparation and testis varies widely from all the other preparations.

Because of the anomalous viscosity, i.e., the dependence of the specific viscosity upon the shear gradient, the values obtained for the intrinsic viscosity cannot be used to obtain the axial ratio by means of the Simha equation. At zero shear gradient, it is expected that the differences in intrinsic viscosity between different preparations become much larger.

The values of the intrinsic viscosity determined here are approximately 1/3 of that found by Pouyet (45). Pouyet, using a special Couette viscometer, found that the intrinsic viscosity in the limit of zero velocity gradient was 62.

PART IV

STREAMING BIREFRINGENCE STUDY OF SODIUM DESOXYRIBONUCLEATE

Theoretical. The following section is a brief discussion of the laws governing the rotational motion of large particles under the influence of a velocity gradient.

Consider a solution of large asymmetric molecules which is so dilute that the interaction of these molecules is negligible. The rotational motion of these molecules is described by use of the rotational diffusion coefficient, θ

$$\theta = \frac{kT}{\zeta} \tag{1}$$

where k is the Boltzmann constant, T the absolute temperature, and ζ , the rotational friction constant, is the torque necessary to maintain unit angular velocity.

In the two dimensional case, if Δn is the number of molecules per unit volume whose fixed axes lie between the angles ϕ and $\phi + \Delta\phi$ from an axis fixed in space, a distribution function may be defined by

$$\rho(\phi) = \lim_{\Delta\phi \to 0} \frac{\Delta n}{\Delta\phi} \tag{2}$$

The two laws of rotary diffusion are

$$dn = -\theta \frac{d\rho}{d\phi}\, dt \tag{3}$$

$$\frac{\partial \rho}{\partial t} = \theta \frac{\partial^2 \rho}{\partial t^2} \tag{4}$$

where t is the time. These laws are analogous to Fick's laws for translational diffusion.

θ is related to molecular dimensions. For a prolate

ellipsoid with semi-major and semi-minor axes a and b, the rotational diffusion coefficient is given by an equation derived by Perrin (46).

$$\theta = \frac{3kT}{16\pi\eta a^3}\left(2\,\mathrm{Ln}\,\frac{2a}{b} - 1\right) \qquad (5)$$

where η is the viscosity of the solvent.

When a solution is subjected to a velocity gradient, the degree of orientation of the solute particles is dependent upon the balance between the orienting force of the velocity gradient and the disorienting force of rotational diffusion. Consider an idealized system of long rods suspended in a liquid which is subjected to a velocity gradient. In the absence of thermal agitation, the rods tend to orient parallel to the streamlines in the liquid. Thermal forces, however, impart random rotations to the molecules. Besides reducing the extent of orientation, rotational Brownian motion influences the direction of maximum orientation. Particles which rotate away from the streamlines against the velocity gradient are retarded; those rotating with the velocity gradient are accelerated. At equilibrium, there is a position of maximum orientation which is determined by the relative magnitudes of the rotational diffusion coefficient and the velocity gradient, G. As G increases, the direction of maximum orientation approaches that of the streamlines.

In 1932, Boeder (47) obtained a quantitative solution for this dynamic stytem in the two dimensional case. The increase in the distribution function with time due to the velocity gradient alone is given by

$$\frac{\partial \rho}{\partial t} = -\frac{\partial(\rho\omega)}{\partial \phi} \qquad (6)$$

where ω is the angular velocity of the particles. The total value of $\frac{\partial \rho}{\partial t}$ is the sum of orienting and disorienting forces, and from equations (4) and (6),

$$\left(\frac{\partial \rho}{\partial t}\right)_{\text{total}} = \theta \frac{\partial^2 \rho}{\partial \phi^2} - \frac{\partial (\rho \omega)}{\partial \phi} \qquad (7)$$

In 1923, Jeffery (48) obtained an expression for the angular velocity of a particle in a velocity gradient. Integrating the above differential equation (7) for the steady state $\left(\frac{\partial \rho}{\partial t} = 0\right)$ and substituting Jeffery's value for $\omega (\omega = -G \sin^2 \phi)$ one arrives at the expression

$$\frac{\partial \rho}{\partial \phi} + a \sin^2 \phi = \text{constant} \qquad (8)$$

where $a = \frac{G}{\theta}$. In 1939, Peterlin and Stuart (49) obtained a solution for the three dimensional case. Their solution, however, was useful for only very small values of a corresponding to low values of G. For higher values of a , their solution was given in terms of a slowly converging infinite series. In 1949, Scheraga, Edsall, and Gadd (50) employed a computing machine to obtain the solution for all values of a and various axial ratios, a/b. This computation is of great value for the interpretation of data at higher velocity gradients.

With a streaming birefringence apparatus, one obtains a measure of the maximum of the distribution function ρ . Streaming birefringence is most conveniently observed when the solution to be considered is placed in the annulus between two concentric cylinders and viewed with light traveling parallel

to the cylinder axis. If crossed polaroids are placed above and below the stationary concentric cylinders, the annular space is dark. On rotating one of the cylinders, however, the annular field becomes light except for a dark cross called the cross of isocline (figure 4). This cross of isocline is defined by an angle, χ , the extinction angle, which is the smaller angle between the arms of the cross and the transmission plane of either the polarizer or analyzer. A flowing solution has the appearance of a uniaxial sphero-crystal, i.e., one in which the optic axis makes a constant angle with the radius vectors of the cylinder.

To simplify the theoretical discussion, elongated molecules in solution are represented by geometrical and optical ellipsoids of revolution. In figure 4, the lines represent both the geometric and optic axes of the particles. Polarized light traveling parallel or perpendicular to the optic axes passes through the solution without rotation of its plane of polarization. Polarized light passing through the solution at points where particles are oriented at an angle to the plane of polarization emerges as elliptically polarized light. The arms of the cross of isocline represent regions in the solution at which the optic axes of the ellipsoidal particles is parallel or perpendicular to the plane of polarization. It can be seen from figure 4 that the angle of isocline, χ , is equal to the angle between the particle optic axis and the streamline at that point.

Since the angle, χ , is related to the maximum value of

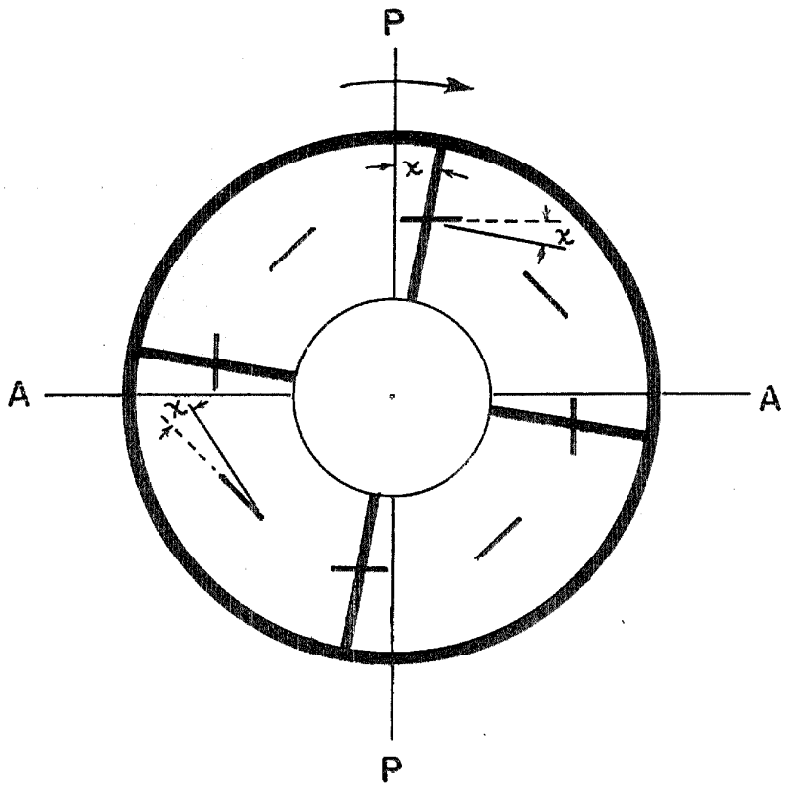


FIG.,4

View of the birefringent medium in the annular gap between the concentric cylinders. The cross of isocline is shown at an angle with respect to the plane of the polarizer for a rotating outer cylinder. The orientation of eight rod-like particles at an angle with respect to the stream lines at high velocity gradient is also indicated.

(Plane of polarizer - PP, analyzer - AA)

the distribution function, it is a function of G and θ . For a given molecule, at low G, the angle, χ , tends toward 45°, i.e., complete randomness. As G increases, χ decreases and asymptotically approaches 0°. For this ideal system of non-interacting, rigid, ellipsoidal particles, the behavior of the extinction angle as a function of α is shown in figure 5.

In addition to the change of χ with G., one can also measure the change in the birefringence, Δn of the system. Peterlin and Stuart obtained the equation

$$\Delta n = \frac{2\pi V}{n_o} \left(g_1 - g_2\right) f\left(\alpha, a/b\right) \qquad (9)$$

where n_o is the refractive index of the solvent, V is the volume fraction of solute, g_1-g_2 is the optical anisotropy factor, which includes both the intrinsic birefringence of the particle and the birefringence brought about by parallel alignment of particles, and $f(\alpha, a/b)$ is an orientation factor, which increase from 0 asymptotically toward 1 as the orientation of the particles increases. f as a function of α and a/b has been tabulated by Scheraga, Edsall, and Gadd (50), and has the form indicated in figure 6. The increase in f and, therefore, Δn varies linearly with α for small values of α and approaches a saturation value for large α .

From measurements of χ , the rotational diffusion coefficient can be obtained. By using equation (5) and an approximate value of a/b, the length of the particle, $2a$, can be calculated.

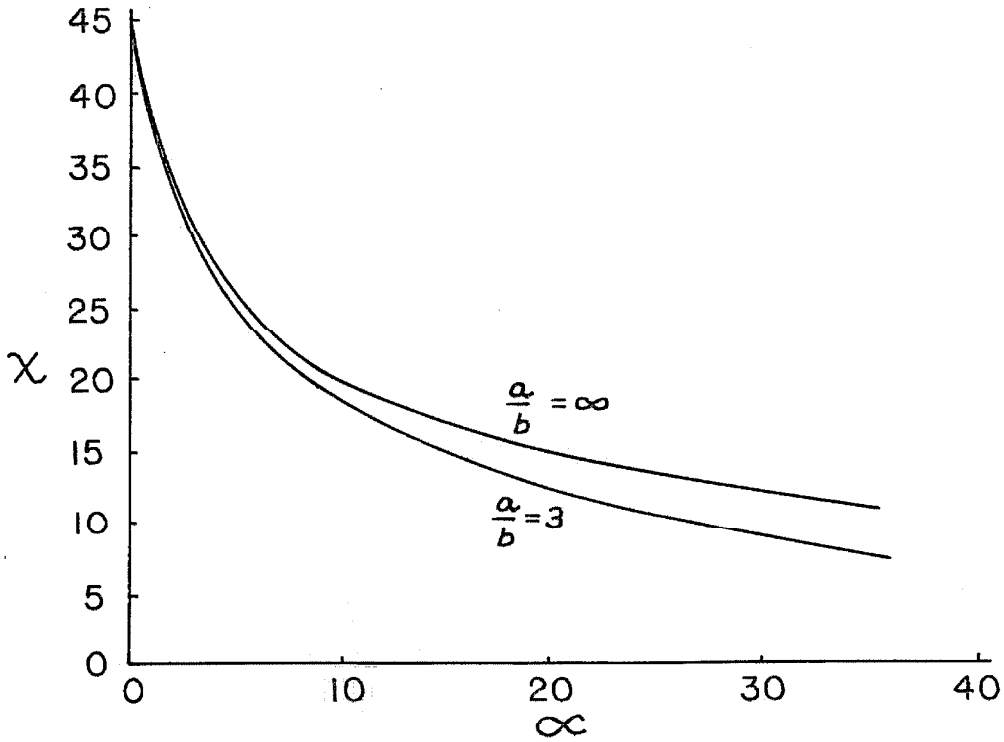


FIG., 5 EXTINCTION ANGLE χ vs. $\propto$. AXIAL RATIO = $\frac{a}{b}$

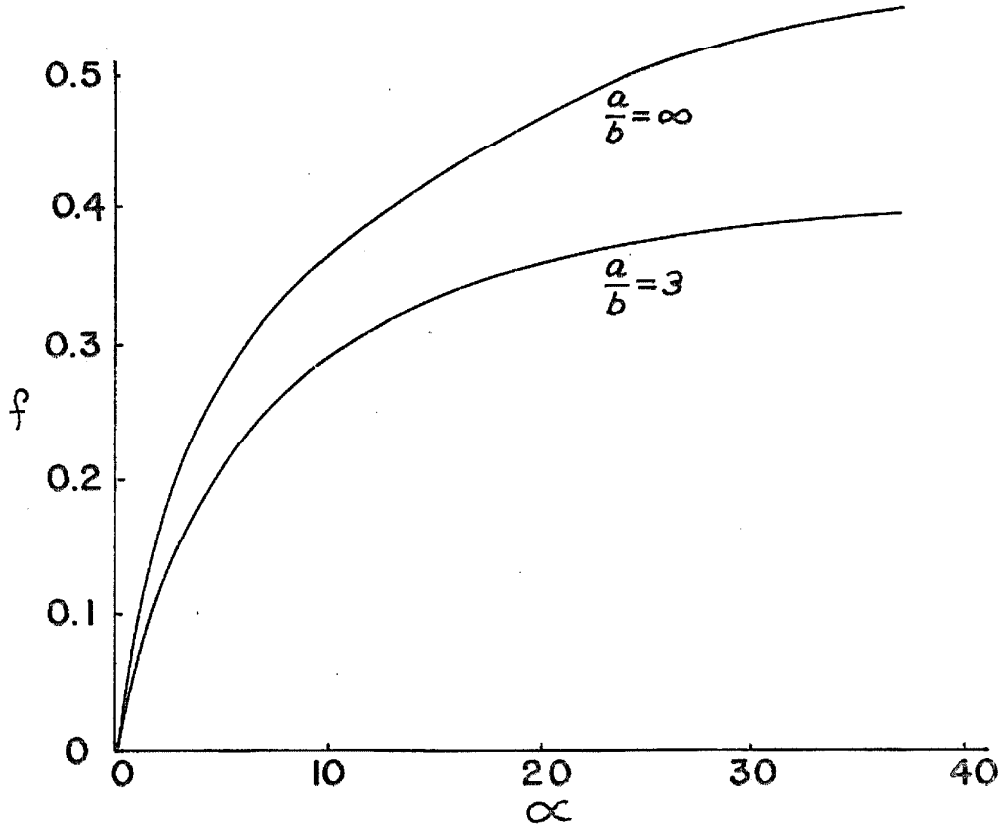


FIG., 6 ORIENTATION FACTOR f vs. $\propto$

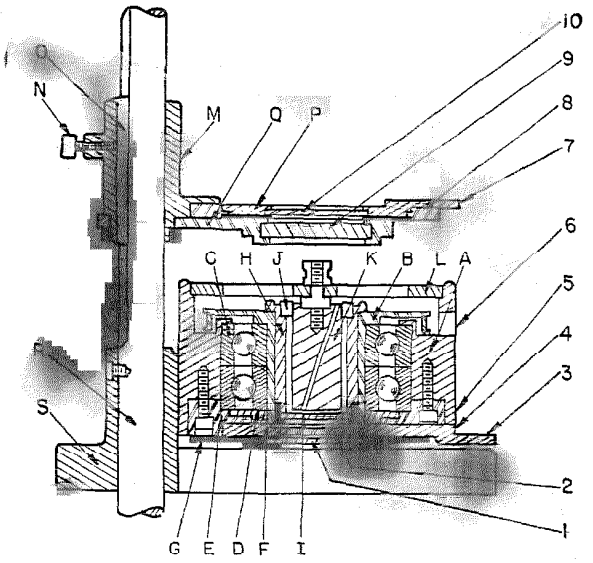
It should be noted that equation (5) is very insensitive to variations in a/b, since the length varies as the cube root of the logarithm of the axial ratio. For example, if a/b = 250, changing this ratio by 100% changes the calculated length by approximately 4%.

Experimental Apparatus. The double refraction of flow instrument used in these studies has been fully described in a recent publication (51) and is consequently described only briefly here. It consists of two vertically mounted concentric cylinders, an inner stator and outer rotor. A collimated beam of light is passed up through the annular space between the cylinders which is filled with the solution under investigation.

A detailed figure describing the apparatus is included here (figure 7) taken from the paper of Edsall, Rich, and Goldstein. A zirconium arc is used as a point source of light, and a large telescopic objective lens is used for collimation. The light passes through the polarizer (1) in figure 7, then through a reticle (2) which has ruled cross hairs. A glass-bottomed bakelite cup (H) is positioned in the center of a steel rotor (B) so that the plane polarized light passes up between it and a central bakelite stator (K). An isotropic annular ring (J) eliminates the meniscus at the top of the annular space containing the solution. As the light emerges from the cup, it passes through a quarter wave plate (9) and a rotatable analyzer (10), after which it is seen by the operator.

The rotor is driven through a belt drive by a variable speed motor operated by a circuit designed to maintain constant torque.

The dimensions of the apparatus are given in Table V.



CROSS SECTION OF THE CONCENTRIC CYLINDER APPARATUS (51)

FIG. 7

Table V

Inner stator radius	R_1	1.0 cm.
Rotor cup radius	R_2	1.1 cm.
Height of solution in rotor cup	L	4.0 cm.
Gap (R_2-R_1)	d	0.1 cm.

Above a critical velocity gradient (which is determined by the dimensions of the machine), turbulent flow develops.[13] For the dimensions cited in Table V, the critical gradient is 3900 sec^{-1}(51). In this study, rotor speeds up to 24 per second were employed. The maximum velocity gradient, given by

$$G = \frac{R_1\omega}{d} \simeq \frac{R_2\omega}{d} = \frac{2\pi N\bar{R}}{d} \qquad (10)$$

is 1585 sec^{-1} where N is revolutions per second, ω angular velocity of the rotor, and $\bar{R}$ is $\frac{R_1-R_2}{2}$.

The extinction angle, χ, is measured when the polarizer and analyzer are crossed. The four arms of the cross of isocline are observed when the rotor is in motion and the reticle is rotated so that its cross hairs coincide with the arms of the cross of isocline. The angle is read on a graduated scale ruled on the rotor housing.

The birefringence in the system is measured by a quarter wave plate used as a Senarmont compensator. The solution is observed at 45° from the arm of the cross of isocline, at which point the optic axis of the flowing system is oriented

---

[13]There is no adequate treatment for turbulent flow.

at 45° from the plane of the polarizer. Light emerging at this point is elliptically polarized. When this light is passed through the quarter wave plate, which is oriented parallel to the plane of polarization, the elliptically polarized light is converted into plane polarized light. The angle which this plane polarized beam makes with the transmission plane of the polarizer is measured with the analyzer. On rotating the analyzer by this angle, Δ, the field at 45° becomes dark if the original light beam is monochromatic. Δ is equal to $\frac{1}{2}$ of the phase difference and related to Δn by the equation

$$\Delta n = \frac{\lambda \Delta}{180 L} \qquad (11)$$

where the wave length, λ, and path, L, both have the same units. If white light is used, a series of interference colors is observed. The angle of rotation of the analyzer can be calibrated for a given wave length in terms of these colors. In these studies, the wave length was 5200 $\mathring{A}$ and L was 4.0 cm. Hence,

$$\Delta n = 7.22 \cdot 10^{-8} \Delta \qquad (12)$$

Since small amounts of birefringence are measured, it is imperative that all glass be isotropic. Small amounts of birefringence seriously disturb the measurement of Δ. The lower limit of the measurements of Δn in this apparatus is approximately 3×10^{-7}, which corresponds to $\Delta \leq \frac{1}{2}^{\circ}$. When Δ is small ($\leq 1^{\circ}$), however, the measurement of χ is very difficult and the precision of the measurements is poor.

Very small amounts of birefringence in the system distort the cross of isocline so that the arms are not exactly perpendicular to each other. To overcome this difficulty, readings were initially taken in all four quadrants for both senses of rotation. This gave an average measure of χ which was assumed to represent the actual value of χ.

An experiment was undertaken to determine the relation between the value of χ determined from measurements in one quadrant and the actual value of χ determined from measurements in four quadrants. To determine the actual value of χ, three measurements were taken at each arm of the cross for both senses of rotation. The average of these 24 measurements gave the actual value of χ, which was compared with the mean value of χ obtained by measurements on one arm of the cross of isocline for both senses of rotation. Twenty-nine such comparisons were made. The differences between the actual value of χ and the mean χ on one particular arm of the cross were positive and negative in random fashion, suggesting that no constant apparatus error was involved. The standard deviation of this mean value of χ from the actual value of χ was 0.45°, which is a measure of the accuracy of readings made on this arm of the cross. Thereafter, all measurements were made on this one arm of the cross with the accuracy represented by this standard deviation, $\sigma = 0.45^{\circ}$.

In measurements of extinction angle and birefringence, constancy of temperature is desirable. Since the apparatus was not designed to operate in a constant temperature bath, however, all measurements were made at room temperature ($22-25^{\circ}$

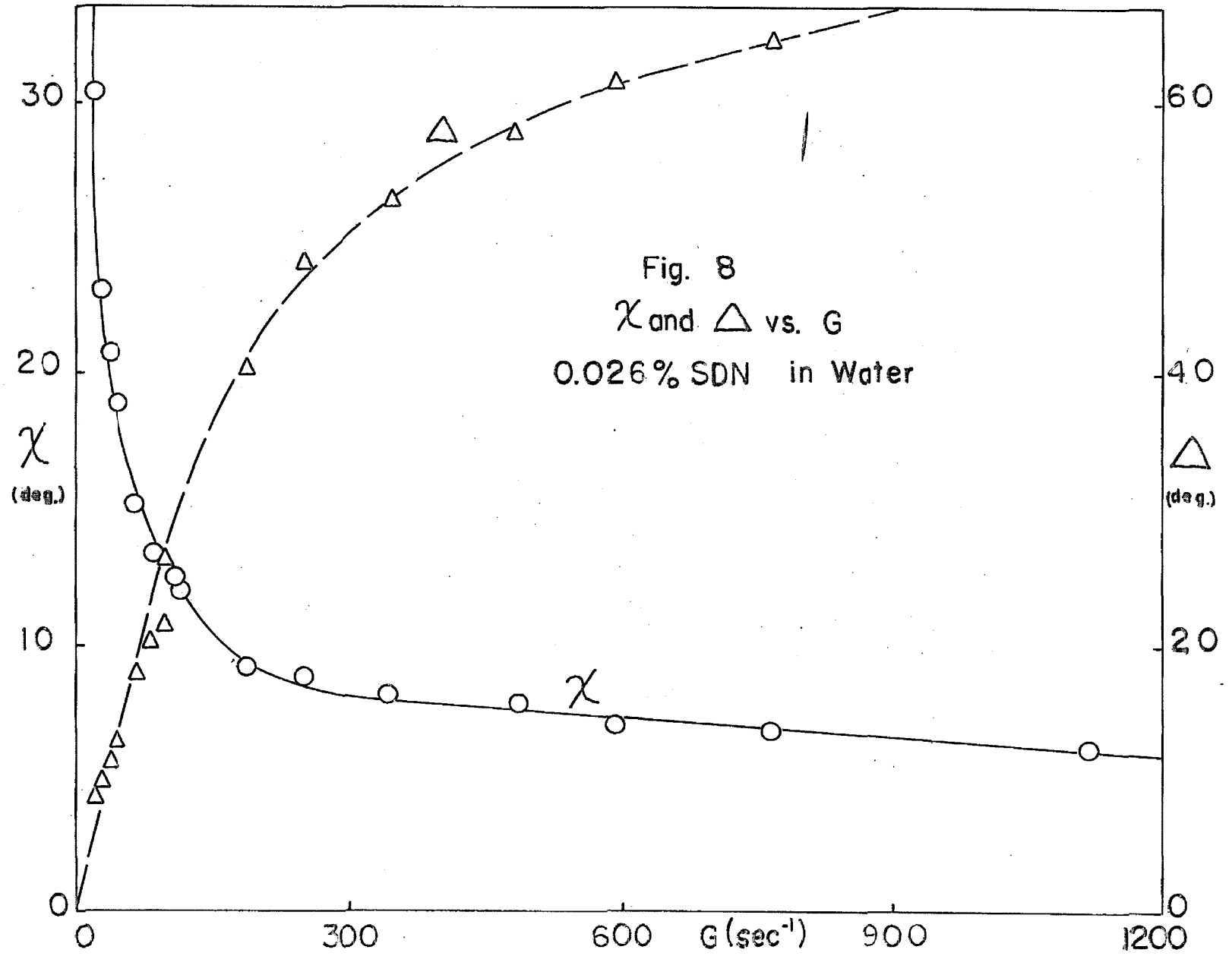


Fig. 8
χ and Δ vs. G
0.026% SDN in Water

C.). Temperature rise in the apparatus due to heating effects has been shown to be negligible (51).

Experimental Results of Streaming Birefringence Study. The initial streaming birefringence work was carried out with distilled water solutions of SDN. Figure 8 shows a typical curve of χ versus G and one of Δ versus G. The characteristic decrease of χ with increasing G resembles that of a theoretical curve of χ versus α (figure 5) where α is proportional to G at constant θ. The curve showing the increase of Δ with increasing G has the form of the orientation factor, f, versus G curve (figure 6).

Further experiments with solutions of SDN in distilled water showed strong concentration dependence. The extinction angle curves (figure 9) continued to rise even in concentrations as low as 0.002%, which is the lowest concentration measurable in the instrument. This limit is imposed by the limiting birefringence which the apparatus can detect.

The origin of this concentration dependence is twofold, First, large molecules interact sterically, impeding their rotational motion. Secondly, electrostatic repulsions between molecules are very pronounced in solutions of polyelectrolytes at low ionic strength. The polyelectrolyte character of nucleic acids has been shown by many workers. These molecules in solutions at pH 6 exist as strongly charged anions, as shown by electrometric titrations (52). The primary phosphate groups are largely, if not entirely, ionized at neutral pH values.

In order to decrease the electrostatic interactions,

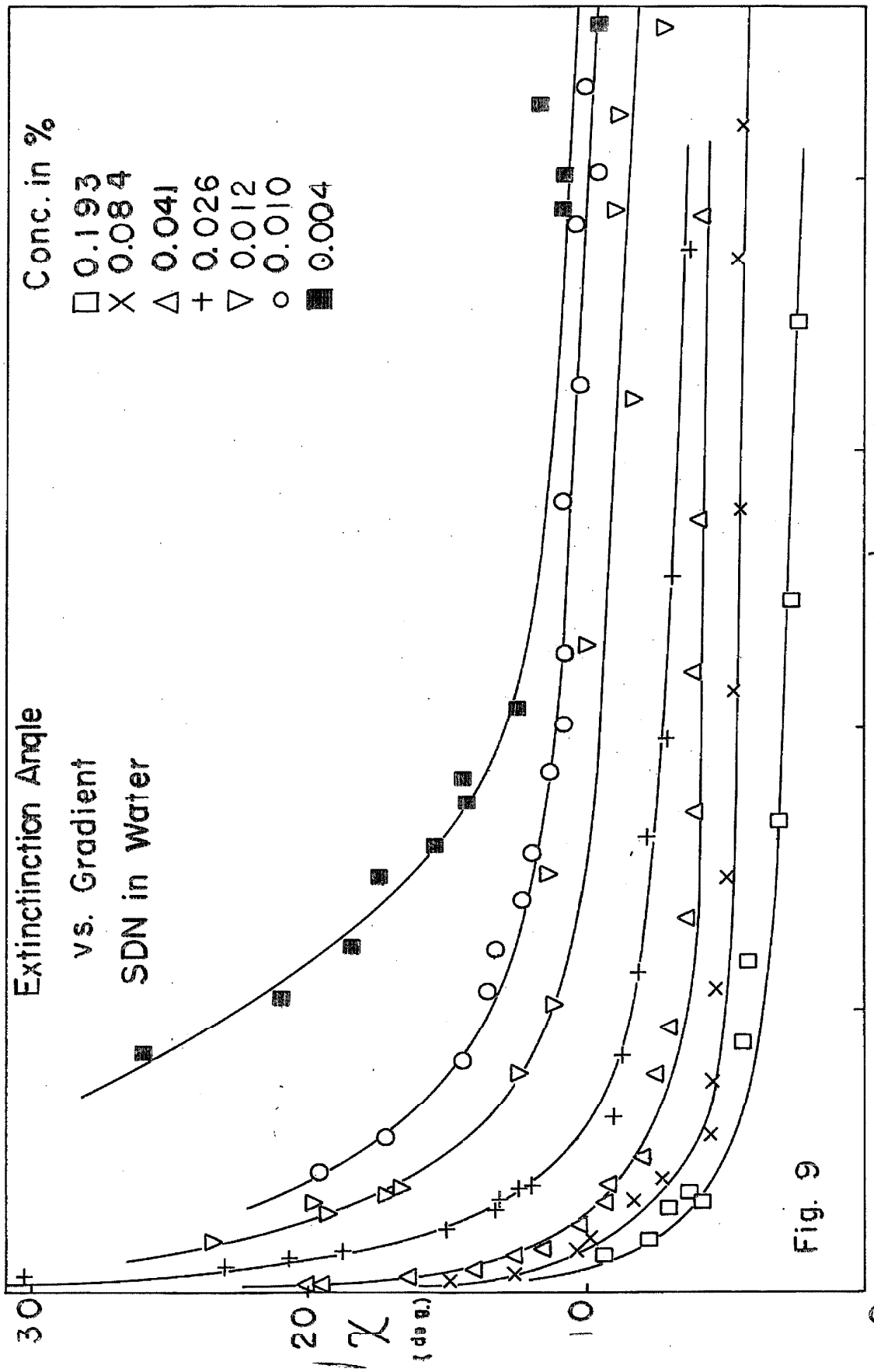

Extinction Angle
vs. Gradient
SDN in Water
Conc. in %
□ 0.193
× 0.084
◁ 0.041
+ 0.026
▷ 0.012
○ 0.010
■ 0.004
χ (deg.)
G (sec⁻¹)
30
20
10
0
300
600
900
1200

Fig. 9

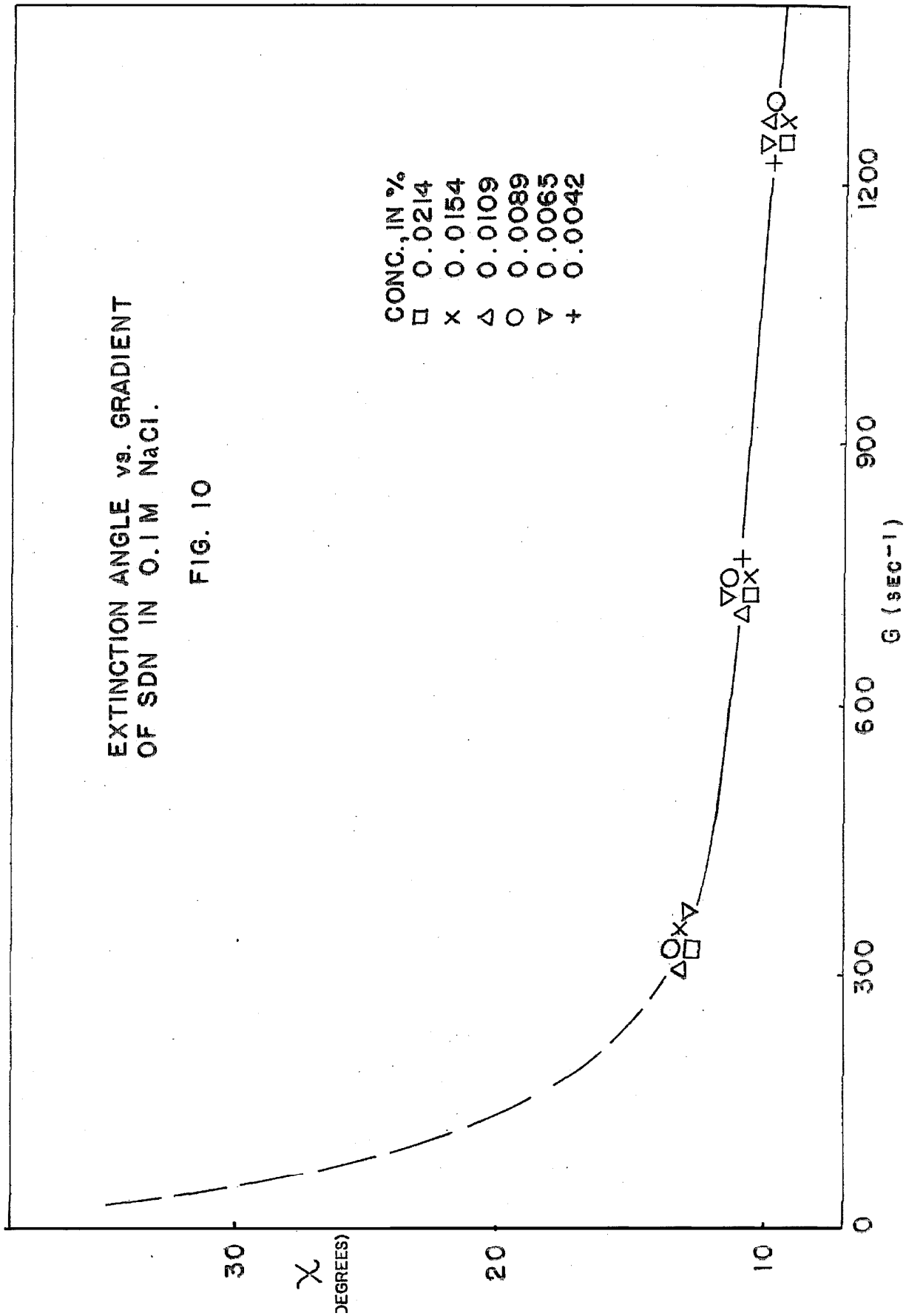

EXTINCTION ANGLE vs. GRADIENT
OF SDN IN 0.1 M NaCl.
CONC., IN %
□ 0.0214
× 0.0154
◁ 0.0109
○ 0.0089
▷ 0.0065
+ 0.0042
χ (DEGREES)
30
20
10
0
300
600
900
1200
G (sec-1)

FIG. 10

solutions of SDN were made up in 0.1 M NaCl. The χ versus G curves in figure 10 show that, in 0.1 M NaCl, a region of concentration independence was reached ranging from concentrations of 0.02 to 0.004%.

In figure 11 are plotted curves of Δ versus G for various concentrations of SDN in 0.1 M NaCl. Figure 12 is a plot of $\frac{\Delta}{c}$ versus G for these same solutions. These curves show the general form of the dependence of Δ upon concentration. From equation (9), it can be seen that $\frac{\Delta}{c}$ is proportional to the product of the orientation factor and the optical factor. For an ideal, monodisperse system containing freely rotating, rigid molecules in a region of concentration independence, the curves of $\frac{\Delta}{c}$ would be superposed upon one another to give one curve. This is largely the case in figure 12. The departure from ideality is shown by the slight spread in the points.

In order to test the reproducibility of the thymus preparations, various birefringence studies were carried out and are plotted in figure 13. Thymus preparations 1-3 gave identical extinction angle curves at concentrations of 0.008% in 0.1 M NaCl. To determine whether all readings were made in the region of concentration independence, some dilutions were made to concentrations of 0.005%. Readings plotted at this concentration gave the same results as did those made on the more concentrated solutions. This substantiates the fact that all readings were made in the region of concentration independence. The shape of the extinction angle curves is discussed below.

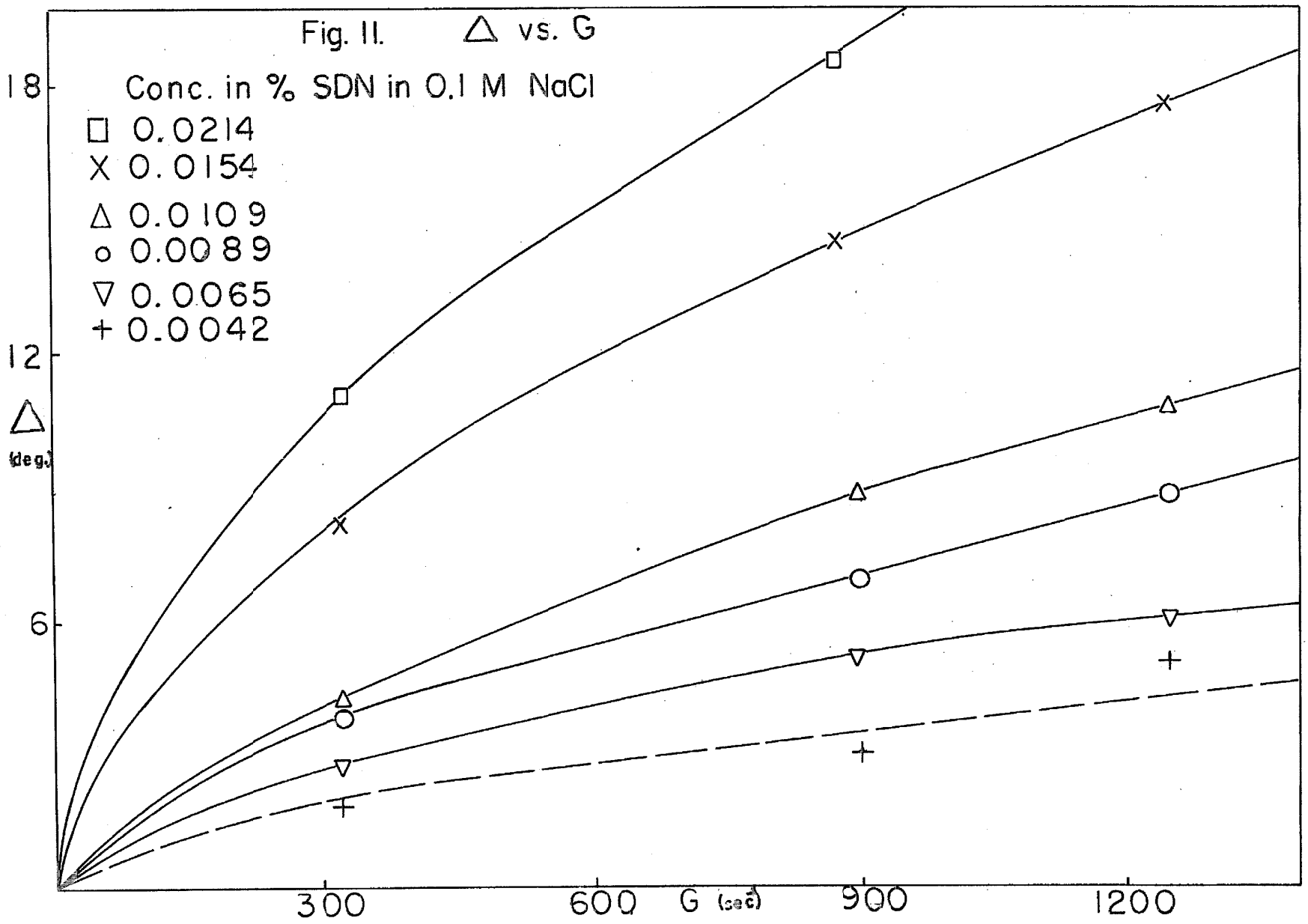


Fig. II. △ vs. G
Conc. in % SDN in 0.1 M NaCl
□ 0.0214
X 0.0154
△ 0.0109
o 0.0089
▽ 0.0065
+ 0.0042
18
12
6
△ (deg.)
300
600
G (sec)
900
1200

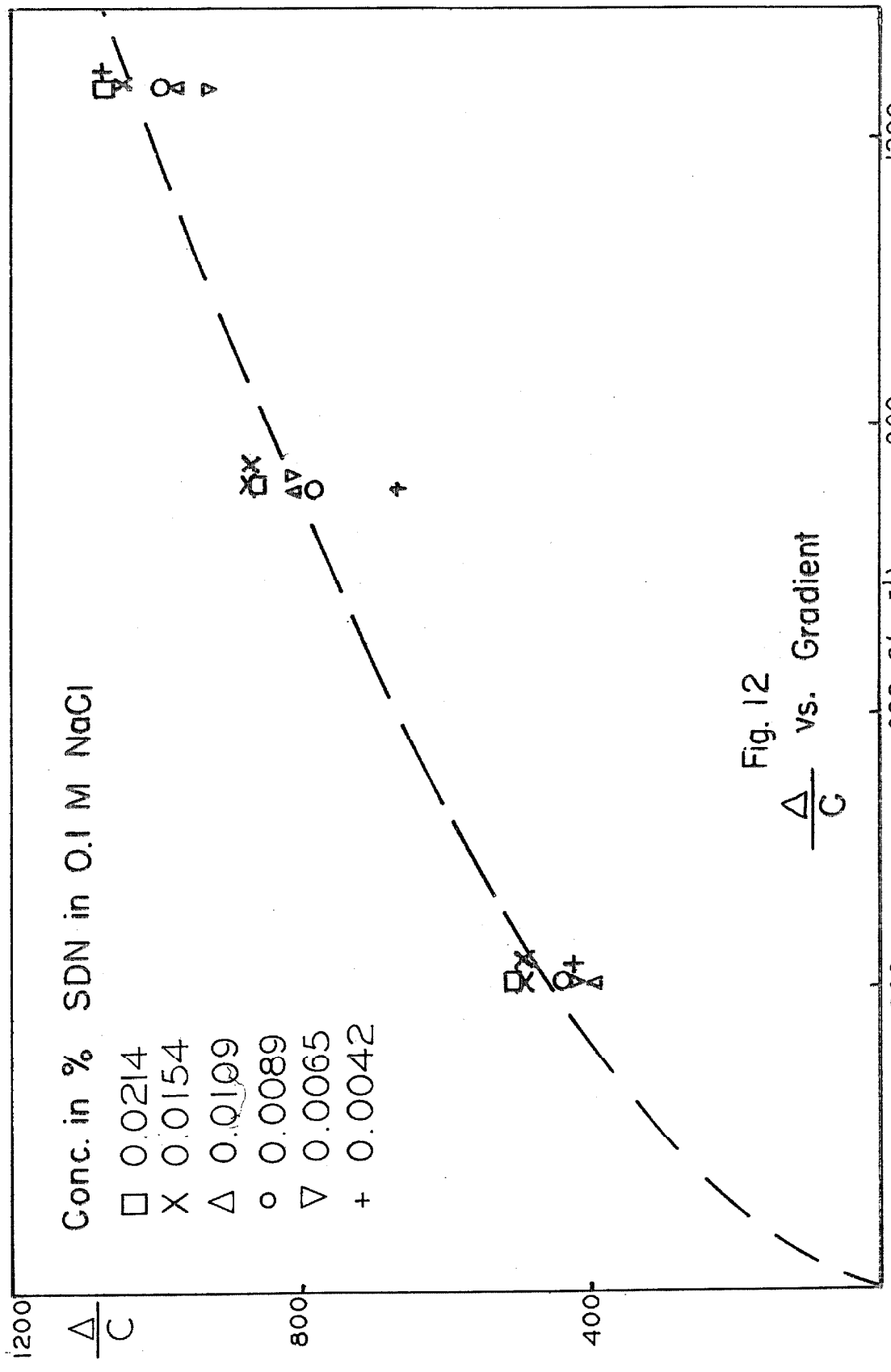
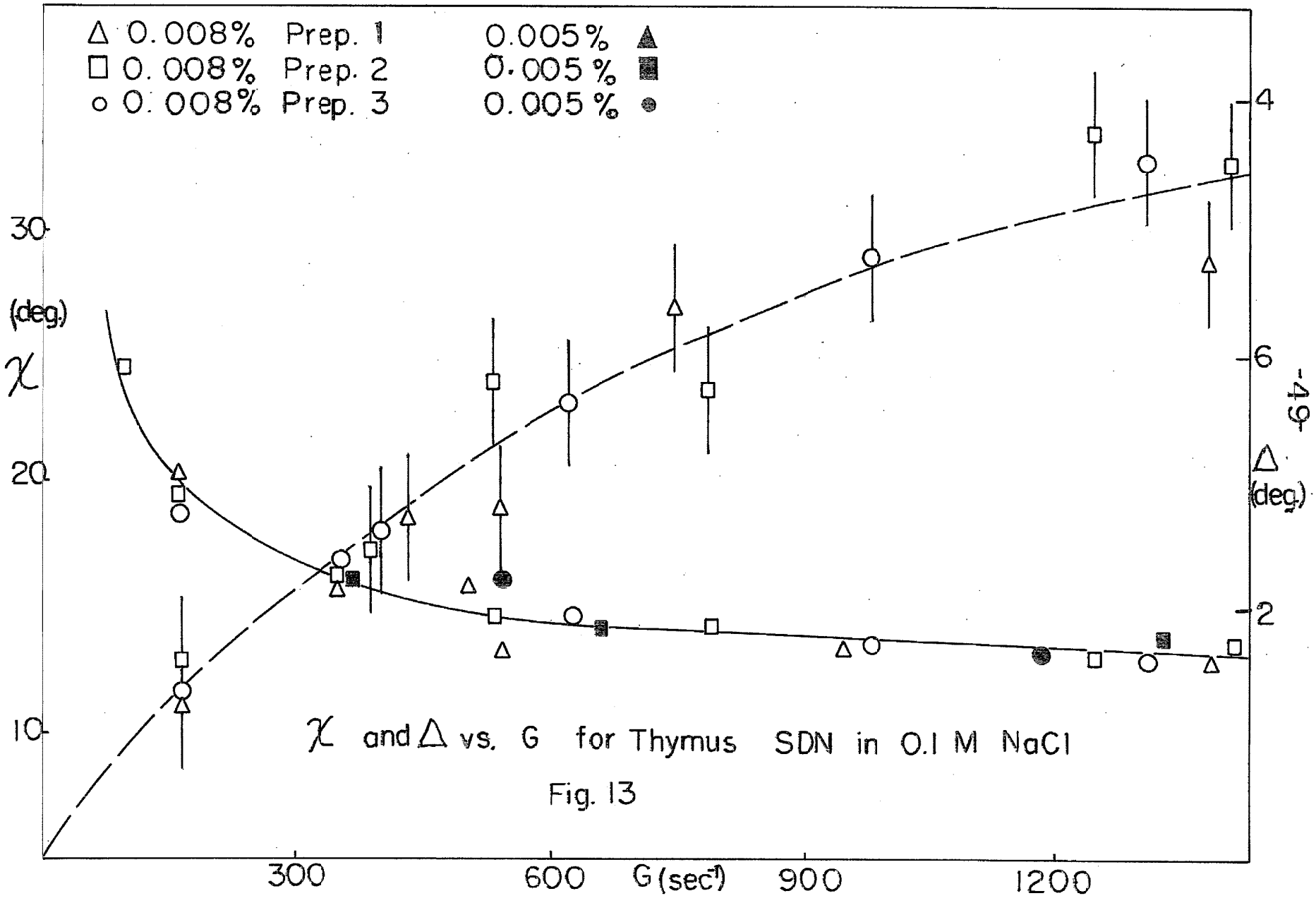


Fig. 12
$\frac{\Delta}{C}$ vs. Gradient



χ and Δ vs. G for Thymus SDN in 0.1 M NaCl

Fig. 13

Table VI

Prep.	Conc. (%)	G (sec^{-1})	χ_{mean} (deg)	σ (deg)	α	θ (sec^{-1})	l/A
1	0.008	172	20.2	.75	9.35	18.4	11,050
	0.008	344	15.3	.73	16.7	20.6	10,550
	0.008	500	16.0	.68	15.2	32.9	9,010
	0.008	540	13.1	.25	18.7	28.9	9,500
	0.008	952	13.2	.72	18.5	51.6	7,580
	0.008	1384	12.7	.20	19.7	70.3	7,060
2	0.008	164	19.2	.95	10.4	15.8	11,640
	0.008	350	16.3	.73	14.6	24.0	10,200
	0.005	372	15.8	.50	15.6	23.8	10,140
	0.008	532	14.5	.71	18.8	28.3	9,560
	0.005	660	14.0	.85	20.0	33.0	9,090
	0.008	792	14.3	.73	19.3	41.0	8,450
	0.008	792	*15.0	--	17.5	45.3	8,190
	0.008	792	*13.6	--	21.2	37.4	8,520
	0.008	1256	13.0	.42	23.3	53.9	7,730
	0.005	1328	14.2	.89	19.6	67.9	7,150
	0.008	1415	13.3	.61	22.2	63.8	7,290
	0.008	1415	*13.9	--	20.3	69.8	7,080
	0.008	1415	*12.7	--	24.6	57.6	7,550
3	0.008	164	18.7	.71	10.9	15.0	11,810
	0.008	164	*19.4	--	10.2	16.1	11,550
	0.008	164	*18.0	--	11.9	13.8	12,150
	0.008	356	17.0	.55	13.4	26.6	9,760
	0.005	514	16.5	.69	14.2	36.2	8,810
	0.008	622	15.9	.55	15.4	40.4	8,500
	0.008	982	13.6	.11	17.5	56.1	7,513
	0.005	1184	13.1	.55	18.7	63.4	7,300
	0.008	1312	12.9	.53	16.8	78.2	6,800

* These values are given to show the effect of the standard deviation in χ on the apparent length.

In table VI are the χ and G values from which the points in figure 13 are plotted. In addition, the standard deviation for each χ is given. This is computed from the equation

$$\sigma = \frac{\sqrt{\sigma_{cl}^2 + \sigma_{ccl}^2}}{2}$$

where σ_{cl} and σ_{ccl} are the standard deviations of the clockwise and counterclockwise sets of readings, respectively. The method of computation of α, θ, and l, the apparent length, is explained on page 54. The values of χ marked with an asterisk are obtained by adding or subtracting the standard deviation from the mean value. This is presented to show the spread of apparent lengths resulting from errors in the measurement of χ at a particular G.

The birefringence curves for the 0.008% solutions (figure 13) are essentially superposed upon one another. Small readings of Δ are difficult. The deviations of the scattered points fall within the expected error spanned by the vertical lines through the points. Two thymus preparations made by N. Simmons[14] using essentially the same preparatory method were obtained. Streaming birefringence studies were undertaken to determine the reproducibility of preparations made in different laboratories. The χ versus G curves (figure 14) for these preparations, designated thymus A and B, are slightly spread apart and are higher than those for thymus preparations 1-3. (A higher curve indicates a larger θ, therefore, a smaller molecular length.)

The χ versus G curves for the spleen and testis preparations are plotted in figure 15. These curves show large and significant differences from those for thymus. The extinction

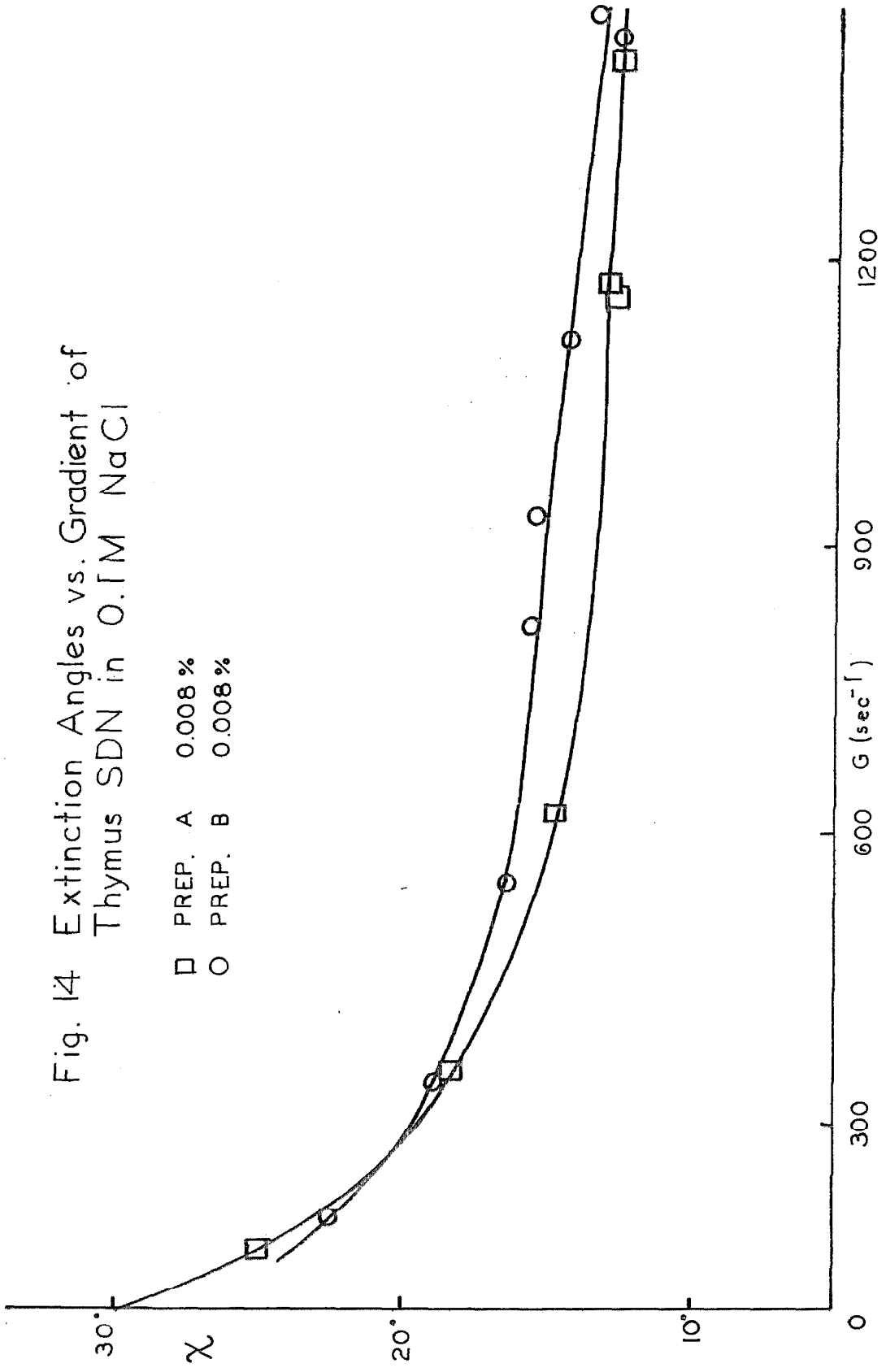


Fig. 14 Extinction Angles vs. Gradient of Thymus SDN in 0.1 M NaCl

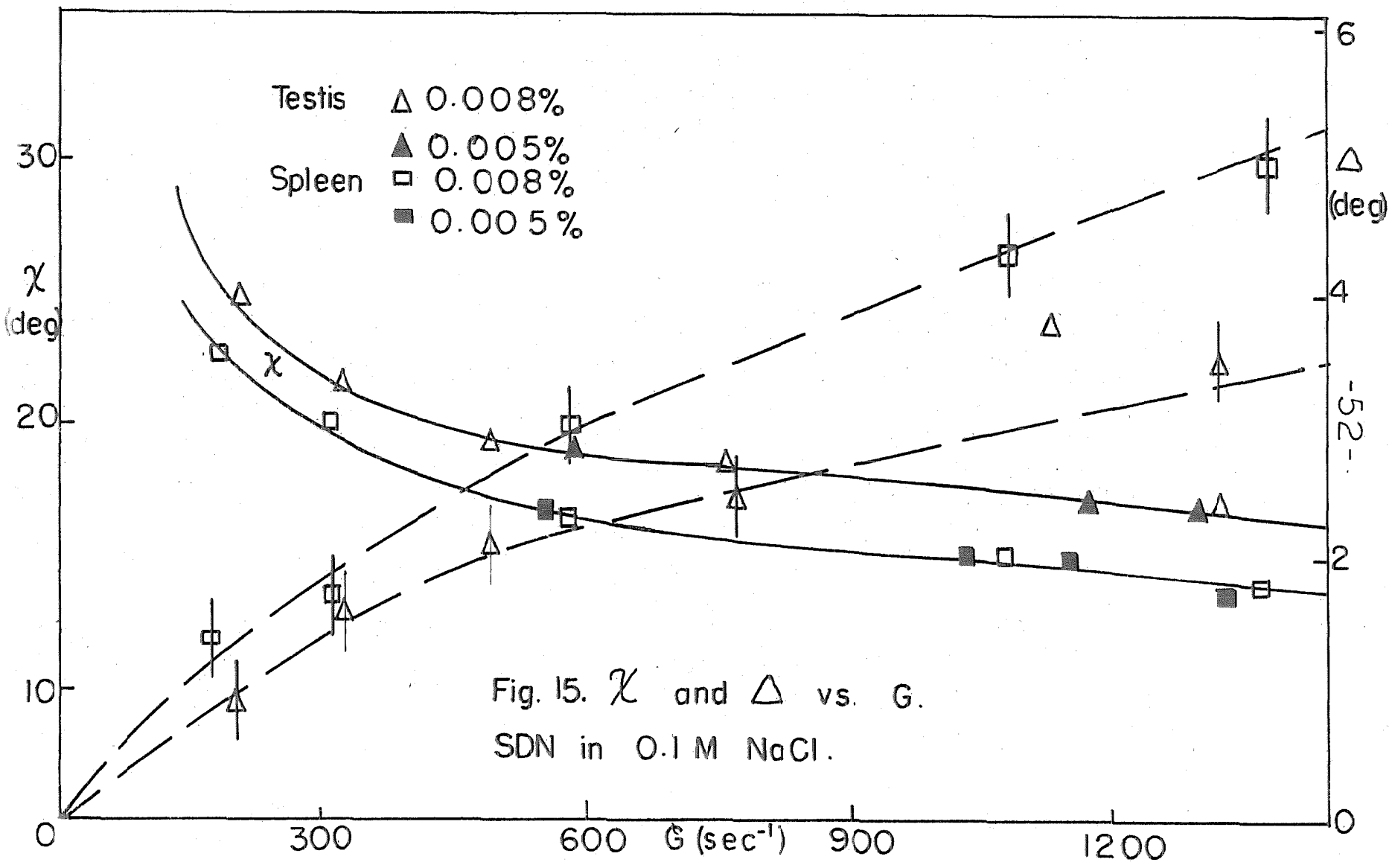


Fig. 15. χ and Δ vs. G.
SDN in 0.1 M NaCl.

Table VII

Prep.	Conc. (%)	G (sec^{-1})	χ_{mean} (deg)	α	θ (sec^{-1})	l (Å)
Spleen	0.008	180	22.5	7.40	24.4	10,050
	0.008	302	20.1	9.44	32.0	9,180
	0.005	548	16.6	14.1	38.8	8,610
	0.008	572	16.5	14.2	40.3	8,500
	0.005	1036	15.2	16.8	61.7	7,370
	0.008	1068	15.1	17.1	62.5	7,340
	0.005	1148	15.0	17.5	65.5	7,230
	0.005	1332	13.3	18.2	73.4	6,970
	0.008	1360	13.8	20.3	67.0	7,180
Testis	0.008	208	24.9	5.94	35.1	8,900
	0.008	324	21.6	9.95	32.6	9,130
	0.008	488	19.4	10.2	47.8	8,050
	0.005	588	19.4	10.2	57.6	7,520
	0.008	760	18.7	10.9	69.4	7,100
	0.005	1160	17.0	13.4	86.5	6,600
	0.005	1308	17.0	13.4	97.6	6,340
	0.008	1320	17.0	13.4	98.5	6,320

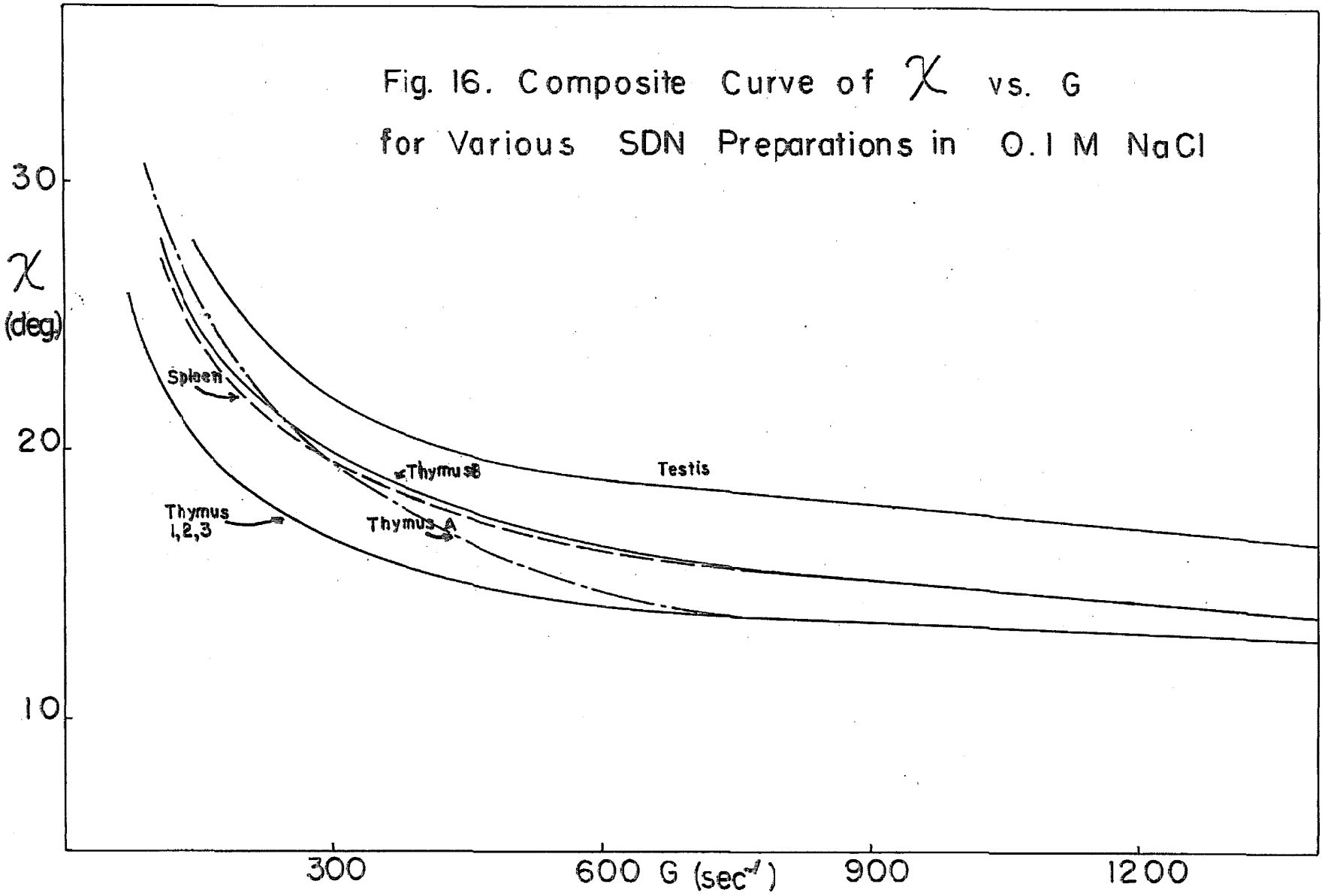


Fig. 16. Composite Curve of χ vs. G
for Various SDN Preparations in O.1 M NaCl
30
χ
(deg)
20
10
Spleen
Thymus B
Testis
Thymus
1,2,3
Thymus A
300
600 G (sec⁻¹)
900
1200

angle curve of testis implies shorter molecules than those found in spleen, and the lower birefringence curve associated with the testis is in agreement with this. The spleen curve for birefringence falls upon that plotted for preparations 1-3 in figure 13.

Table VII contains the χ and G values for the points plotted on the extinction angle curve in figure 15. For each point, an α, θ, and l is also tabulated. It can be seen that, at the same velocity gradient, the apparent length of the spleen molecule is larger than that of testis. (This is discussed further on page 54 ff.)

All of the χ versus G curves of figures 13, 14, and 15 (but not the experimental points) are plotted together in figure 16. The extinction angle curve for spleen falls upon that for Simmons' preparation B. The curve for A falls partly on that for B and partly on the curve for preparations 1-3. The testis curve lies outside the range of all the other curves.

Discussion. Comparison of the extinction angle curves shows that preparations from calf thymus tissue made by a single investigator appear to be reproducible. A semi-quantitative treatment of these data may be made.

For each measurement of χ, there corresponds an apparent rotational diffusion coefficient, i.e., the θ which a monodisperse system would have at this χ and G. By the use of equation (5), this apparent rotational diffusion

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[14]The author wishes to thank Dr. Simmons for kindly supplying these preparations.

coefficient can be converted into an apparent length. The curve of apparent length versus G gives some indication of the distribution of molecular lengths.

The axial ratio used in equation (5) is 250. This estimate is based upon molecular weight (26) and x-ray diffraction data (12). Although this number is chosen somewhat arbitrarily, the value of the length is very insensitive to the choice of axial ratio, as noted on page 36.

Figure 17 is a plot of apparent length versus G for all the preparations.[15] The numbers on the curves are the apparent rotational diffusion coefficients at the various values of G. For thymus preparations 1-3, the range of apparent lengths is 7,500 Å to 11,500 Å. For the testis, the entire distribution is shifted to lower values. It is seen that relatively small changes in the extinction angle curve correspond to large changes in apparent length.

The sequence of these curves agrees with the viscosity measurements. If a rod-like shape is assumed for the SDN molecules and if the only variation between different prepara-

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[15] The values of χ and G used for the calculations were taken from the smooth curves, and, in most cases, are interpolated values rather than experimental points.

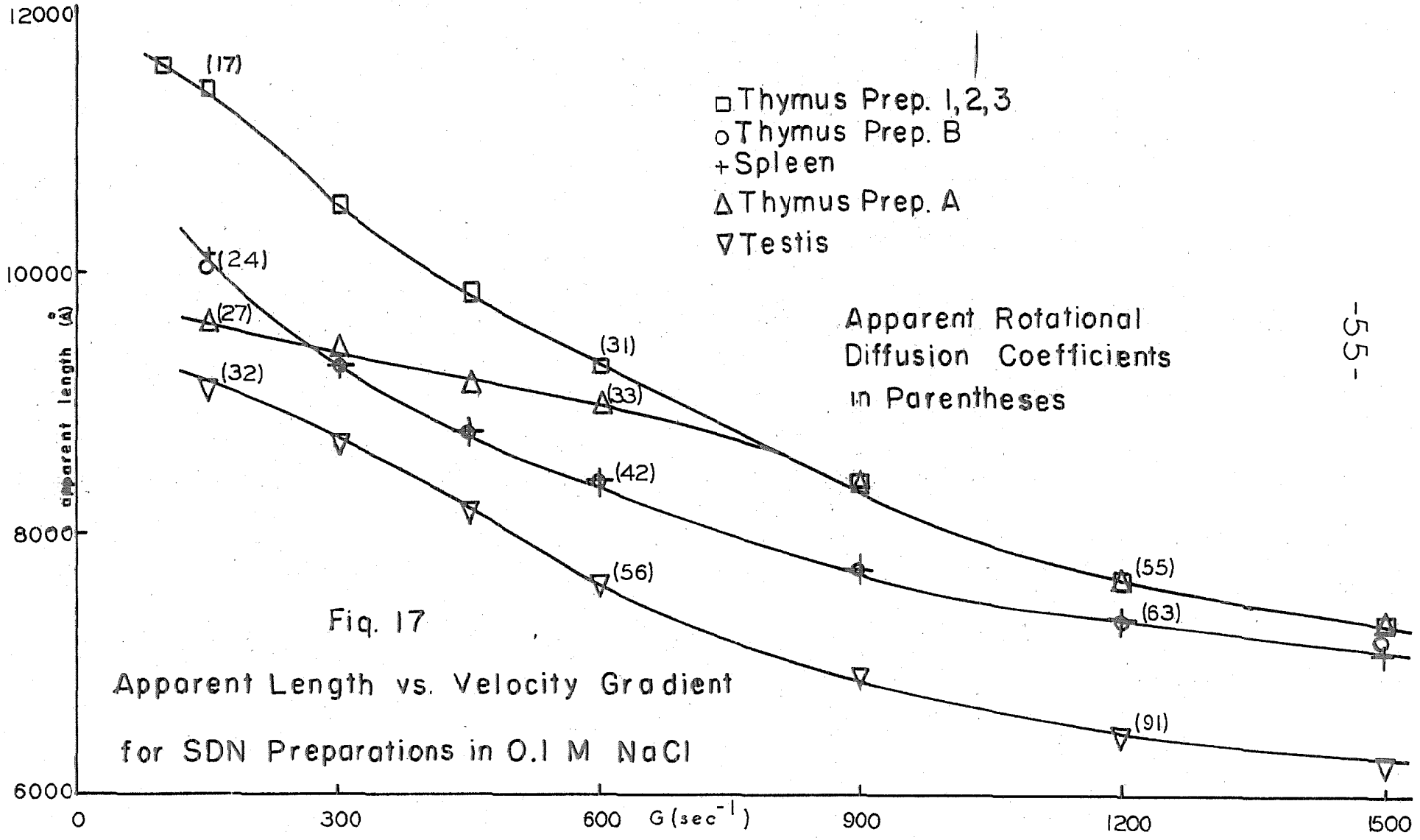


Fig. 17

Apparent Length vs. Velocity Gradient

for SDN Preparations in 0.1 M NaCl

tions is the distribution of molecular length, then the viscosity measurements qualitatively indicate that thymus preparations 1-3 have the longest molecules, spleen is intermediate, and testis has the shortest. (It may be noted that streaming birefringence is a much more sensitive tool than viscosity for examining molecular lengths of ellipsoidal particles.)

Since preparations A and B were prepared from the same tissue as preparations 1-3, it must be assumed that A and B were degraded in the course of their preparation. The influence of this degradation dropped the curve of apparent length (plotted against G) over a wider range of G in preparation B than in A. Since the curve for spleen SDN falls on that of the degraded thymus preparations, the possibility that spleen experienced a similar degradation during its preparation can not be excluded.

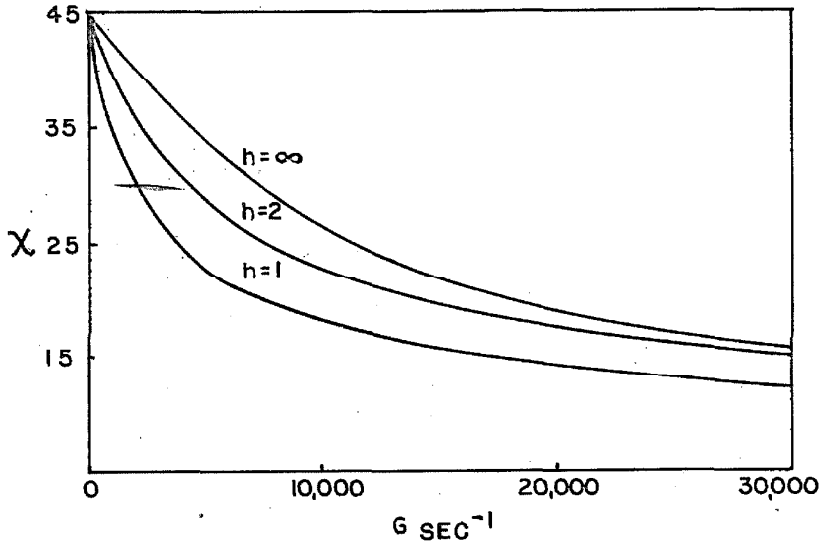
The departure of the testis length distribution from that of the other preparations may imply even further degradation from an initial state described by the curve for thymus 1-3. It may also reflect the fact that testis is a very special tissue from the nuclear point of view, since it contains haploid cells. There is a possibility that the nucleic acid molecule has a different macromolecular form in sperm cells than in the usual diploid tissue cell. This problem may be resolved when preparations of SDN are made from sperm cells by the methods used here.

Various reasons may be advanced for the polydispersity indicated in figure 17. First of all, nucleic acids are believed

to be related to genes. The manifold activities of genes imply that they have different molecular configurations. Therefore, the DNA molecules may be expected to vary not only in detailed molecular structure but also in their macromolecular structure. On the other hand, it is possible that all the nucleic acids from the same species may have the same molecular length, but this has never been demonstrated.

A discussion of polydispersity will shed light on the interpretation of figure 17. Sadron (53) was the first to derive an expression for the extinction angle curves in polydisperse systems. Using artificially prepared polydisperse systems with components of known length, he demonstrated the applicability of his equations (54). Utilizing the complete solution of the Peterlin and Stuart treatment, Scheraga (55) applied Sadron's equations to Gaussian distributions of lengths. His results are shown in figure 18. The curve for h=∞ represents a monodisperse system. The curves for lower values of h indicate the effects of polydispersity. It can be seen that the latter curves show a fairly abrupt change in slope. This change is similar to that exhibited by the curves obtained for the SDN solutions. These curves are interpreted as showing that, at low velocity gradients, the longer molecules are oriented; hence, the extinction angle falls. At higher G values, the shorter molecules orient, and the curve no longer follows the initial course determined by the larger molecules.

It should be emphasized that a given extinction curve can be produced by many different distributions of molecular lengths,



EXTINCTION ANGLE vs. GRADIENT FOR GAUSSIAN DISTRIBUTION OF PARTICLE LENGTHS (55)

FIG. 18

both discrete and continuous. Consequently, no unique interpretation in terms of molecular lengths can be given to the curves obtained in this study. For this reason, only an apparent distribution of lengths can be described.

Another possible explanation for the departure of the extinction angle curves from ideality is that the SDN molecules do not behave as rigid rods in solution. Schwander and Cerf studied the birefringence behavior of SDN in solutions of varying viscosity and came to the conclusion that the DNA molecule is not compressible but acts as a rigid rod (56). Their study did not consider the question of flexibility of this long molecule at high velocity gradients, however. Schwander and Signer (57) made a study of the effect of salt concentration on the extinction angle curves. They found that the curves did not change with salt concentration, and inferred from this that the molecule is fairly rigid. The high concentration dependence of the χ vs. G curves of DNA in water solution obscures the question of shape changes in the presence of small amounts of salt. Nonetheless, the invariance with salt concentration supports the concept of rigidity in the presence of salt.

The difference in the apparent length distributions of preparations 1-3 and of A and B implies that there is an uncontrolled variable in the preparation. Some reflection suggests that the Waring blendor used in dispersing tissue may be important in this connection. In the preparation described here, care was taken to keep the blendor speeds as slow as possible.

This resulted in slower dispersion of tissue and a lower final yield, but gave a reproducible preparation of thymus SDN. Since virtually all preparative methods employ the Waring blendor or some similar instrument, it was decided to investigate the effects on the final product of mixing in the Waring. An experiment was carried out in which a 0.008% solution of thymus SDN was mixed in the blendor at high blade speeds of approximately 40 R.P.S. Aliquots removed from the blendor at various intervals showed a rise in the χ versus G curve, indicating the presence of smaller particles. After 30 minutes, almost all birefringence had disappeared.

Variations in blendor action upon different tissues may account for the different results obtained for spleen and testis. Also, degradation due to blending may account for the variations in results among different investigators.

Comparisons of the results found here with those of previous investigators are essentially a comparison of different preparative methods. Until the recent calculations of Scheraga, Edsall, and Gadd were available, investigators determined one rotational diffusion coefficient and therefore one length by taking the limiting slope of the χ versus G curve as G approached zero. This gave only a measure of the maximum length. Early studies by Signer, Caspersson, and Hammersten (58) yielded a θ of 180, which corresponded to a length of 4500 Å. The Hammersten method of preparation which they used is now generally believed to give degradation. Schwander and Cerf in 1949 (59)

carried out measurements on a calf thymus preparation which gave $\theta = 36.6$, corresponding to a length of 8000 Å. The present investigator replotted their data and found that their preparation closely approached a monodisperse system over a small range of G. In 1951, using essentially the same preparation, Schwander and Signer (57) obtained a curve for a polydisperse system with a maximum length less than 8000 Å. More recently, Steiner (38) characterized a preparation of SDN made by the method of Gulland et al. Although his results showed polydispersity, he claimed that the χ versus G curve closely matched the theoretical curve for a length of 4000 Å.

It should be noted here that no previously published studies have obtained maximum lengths as large as those found in this study. In addition, no earlier studies have attempted to compare the apparent distribution of lengths of SDN made by the same method of preparation in different laboratories or attempted to characterize SDN prepared from different tissues of the same species.

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