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SULFATED AMINOPOLYSACCHARIDES

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This invention relates to a new class of polymeric carbohydrates. More particularly, it relates to polymeric carbohydrates bearing sulfated amino groups.

Agents having the property of prolonging the clotting time of blood in man or other animals are called anticoagulants. Anti-coagulants which may be used systemically have been of great valve to the medical profession in diseases of the heart and blood vessels. Medical interest in anticoagulants is based on the fact that an extremely high percentage of persons in the older age groups die or are crippled by thromobotic episodes. Thus it has been reported by Wright et al. (Amer. Heart P. 36, 801 (1948); Ann. Int. Med. 30, 80 (1949)) that in a study of coronary thrombosis with myocardial infraction, the results indicated that death from coronary thrombosis can be reduced from 23 percent to 13 percent by anticoagulant therapy and, also, that incidence of thromboembolic complications can be reduced from 19 percent to 9 percent. Anticoagulants have been used successfully in the prevention of postoperative thrombosis and embolism, in treatment of thrombophlebitis, as an adjunct to penicillin in treating subacute bacterial endocarditis, in vascular surgery, in frost-bite, and gangrene, and to facilitate replacement of blood in infants with erythroblastosis fetalis.

The most successful clinical anti-coagulant has been the material first prepared in 1916 by Howell and Holt from liver. They called the material heparin. Because of the presence of impurities this heparin was not suitable for medicinal use. The active principle was first separated by Schmitz and Fischer (Ztschr. physiol. Chem. 216, 264 (1933)) and, almost simultaneously, by Charles and Scott (J. Biol. Chem. 102, 425 (1933)). It was further purified by Jorpes, Biochem. J. 29, 1817 (1935); 203 (1942) who identified it as a polysulfuric acid ester of mucoitin, a glucoprotein.

The exact composition of heparin is unknown and it is highly probable that it is not a single compound. Thus Kuizenga and Spaulding (J. Bio. Chem. 148, 641 (1943)) perfected a process for extracting heparin from beef lung tissue; in the process of further purifying relatively pure samples of heparin they separated two fractions of different activities. Furthermore, the heparins obtained from different mammalian species are not identical. The heparin used for human therapy is prepared from ox tissues.

Heparin disappears rapidly from the blood stream, and following its administration the clotting time returns to normal within sixty to ninety minutes. It must, therefore, be administered repeatedly by subcutaneous infusion. However, by incorporating heparin in the Pitkin menstruum (a solution of gelatin, dextrose, glacial acetic acid and water) it has been found that the heparin is absorbed so slowly that an injection of 0.3 gram subcutaneously every two or three days is adequate for the treatment of venous thrombosis or subacute bacterial endocarditis. It has also been reported that heparin can be effectively administered clinically by the sublingual route; from the point of view of the patient this is equivalent to oral administration.

There have been several difficulties attached to the use of heparin. It is expensive because of its source and method of preparation. Thus, Kuizenga and Spaulding reported that under optimum conditions one pound of beef

lung tissues was required to prepare the amount of heparin contained in one U. S. P. ampule. The daily dose is about one or two ampules. Other drawbacks to the use of heparin include the occurrence of toxic reactions, the relatively narrow margin between the therapeutic and toxic dosage, the necessity for biological standardization, and the variability inherent in impure mixtures isolated from natural sources.

Dicumarol (bishydroxycoumarin) is typical of the synthetic anticoagulants of low molecular weight and known structure. Like all such compounds, it suffers from the serious disadvantage that the development of its effect requires 12 to 28 hours and persists for 24 to 72 hours after therapy has been discontinued. This type is thus of no value when immediate increase in clotting time is desired and presents difficulties when the physician desires to terminate or lessen the increase in clotting time.

Ideally, an anticoagulant should fulfill the following requirements:

(1) It should be therapeutically active when administered orally or parenterally—without producing digestive or hypersensitivity reactions.

(2) Its action should be rapid, affecting the clotting tendencies of the blood within one hour.

(3) The dosage should be easily standardized and fairly uniform for a given patient and as between different patients. The action should be predictable in terms of quantitative response.

(4) It should be relatively non-toxic with a wide safety zone between therapeutic effect and toxic damage to important organs. It is obvious that there will always be some hazard from bleeding essential to the very nature of this therapeutic approach. This should not be regarded as a toxic effect but rather as an overextension of the therapeutic effect of the drug.

(5) The action of the drug should be promptly terminated by stopping its administration or by the use of an effective antagonistic agent which in itself is free from undesirable effects.

(6) A test for the activity of this substance should be sufficiently simple to permit its control by the family physician, or, even better, by the patient.

(7) The anticoagulant should be inexpensive.

One object of this invention is to produce a new class of chemical compounds containing members which decrease the clotting tendencies of the blood.

Another object of this invention is to provide inexpensive processes for preparing a new class of chemical compounds containing members which increase the clotting time of blood.

Still another object of this invention is to provide a therapeutic agent whose toxic actions are minor enough to permit its use in clinical medicine.

A further object of the invention is to prepare polymeric carbohydrates bearing —$NHSO_3H$ groups attached to a plurality of carbon atoms and their organic and inorganic salts.

In accordance with this invention, I have discovered a new class of chemical compounds consisting of polymeric carbohydrates bearing —$NHSO_3H$ groups attached to a plurality of carbon atoms and the organic and inorganic salts thereof.

I have also discovered that members of this new class of compounds possess the highly useful property of increasing the clotting time of blood. I have also discovered practical processes for the production of these compounds.

As starting materials, I may use any protein-carbohydrate complexes which contain polymeric carbohydrates yielding, on hydrolysis of the carbohydrate portion, a free or acetylated amino sugar. Examples of these are certain mucopolysaccharides such as hyaluronic acid,

heparin, chondroitin sulfate, micoitin sulfate, hyaluronic acid sulfate, chitin, and certain bacterial polysaccharides, e. g. type I, IV, and XIV pneumococcus specific; and mucoproteins such as ovomucoid, serum mucoproteins, certain hormones of the anterior lobe of the pituitary, e. g. follicle stimulating, luteinizing, and gonadotrophic hormone, cholinesterase, and submaxillary mucin.

Thus, D-glucosamine (chitosamine or 2-amino-2-deoxy-D-glucose) and chondrosamine (2-amino-2-deoxy-D-galactose) are two hexosamines which have been isolated from natural sources. D-glucosamine is the main constituent of chitin. Chondrosamine can be prepared readily from the chondroitin sulfate of cartilage.

Chitin is said to be the most widely distributed polysaccharide of an amino sugar for it occurs in plants, fungi, animals and crustacea, as skeletal material, and it functions as a highly resistant protective substance. Its sole constituent unit is through to be N-acetyl-D-glucosamine, with several hundred of such residues linked through the 1.4 positions to form a linear structure which may be regarded as a 2-acetamido-cellulose. By treatment of chitin with alkali the acetyl groups can be removed and a polyglucosamine formed.

I prefer as starting materials chitin, hyaluronic acid and chondroitin because they are obtained more readily and at nominal expense.

These materials generally occur with the nitrogen atom acetylated. There are numerous methods of N-deacetylating these materials, such as sodium chondroitin sulfate, without seriously degrading the material or causing serious internal structural changes which would prohibit the acceptance of the final product as a synthetic anti-coagulant. In general, alkaline reagents are used such as sodium hydroxide, sodium methoxide, barium hydroxide, and similar compounds of the alkali metals and alkaline earth metals. The deacetylation may be carried out without seriously degrading the product by the use of appropriate alkali treatments; for example a rather highly deacetylated chondroitin sulfate may be obtained by treating 3 grams of sodium chondroitin sulfate (dissolved in 15 ml. of pure water) with a 50% solution of sodium hydroxide in water (35 ml.) at room temperature for 24 to 48 hours. The reaction should be conducted under a nitrogen atmosphere and there is evidence that the addition of a small amount (1.5 ml) of benzyl alcohol to the reaction mixture, or similar such antioxidants, would be beneficial in inhibiting the degradation of the product. In such cases, the product may be isolated by acidification, as with a mineral acid such as hydrochloric acid, subsequent precipitation by the addition of water-soluble organic solvents in which the product is insoluble, such as ethanol. Examples of materials which may be purified for use in subsequent sulfation include the sodium salts of chondroitin sulfuric acid and hyaluronic acid as well as N-deacetylated chitin.

These basic deacetylating agents may alter viscosity, which is indicative of molecular weight and polymer chain length. Since the relative anti-coagulant activity of the compounds of this invention depends upon such factors as the degree of N-sulfation, the degree of O-sulfation, the degree of polymerization and the nature of the polysaccharide, it is obvious that conditions for deacetylation must be chosen with these factors in mind. In general, it is desirable to obtain a maximum degree of N-deacetylation and a minimum of degradation effects or internal chemical changes in the polymers. I do not imply that the N-deacetylation need be absolutely complete.

The final step in the production of the products of this invention consists in sulfating the free amino groups on the polymeric carbohydrates described above. The starting materials comprise polymeric carbohydrates bearing free amino groups and examples have been given above. The usual sulfating agents, e. g. chlorosulfonic acid, sulfur trioxide, addition products of chlorosulfonic acid with pyridine, dioxane, urea or other amides, sodium chlorosulfonate, sulfur monochloride, and sulfur dioxide and pyridine in the presence of copper, a copper salt or iodine, may be used if reaction conditions are adjusted to avoid excessive depolymerization or degradation of the polymer. Solvents known to the art will suggest themselves, such as the use with chlorosulfonic acid of such solvents as ethyl ether, β-chloroethyl ether, ethyl acetate and carbon tetrachloride. The products will vary in activity with such factors as the degree of N-sulfation. The products may contain both acetylated and sulfated amino groups. The product may be useful as produced or may require further purification, e. g. as by dialysis or fractional precipitation. In addition to the sodium salt and the free acid form, it will occasionally be found useful in manufacturing steps, in analytical work or in clinical administration to use other organic or inorganic salts, such as the pyridine salt or salts of aliphatic or aromatic amines or the aluminum, barium or potassium salt. The choice of such salts will depend on many factors related to the intended use, such as decreased toxicity or decreased solubility in water. The methods of preparing and isolating such varying salts will be obvious to those skilled in the art and may include such procedures as precipitation, adsorption and solvent removal or lyophilization. Variations of such procedures, including the use of dialysis will serve to purify the product. It is of course to be understood that the intermediates and final products of the present invention are not expected to be individual chemical entities but will exhibit variations and occur as mixtures after the usual manner of polymers.

When the starting reagent is insoluble in the solvent used, e. g. deacetylated chitin in pyridine, the surface condition of the suspended solid plays an important role during the sulfation reaction. Various methods may be used to produce a colloidal suspension which is readily susceptible to sulfation. One procedure which has been found successful comprises suspending ten parts by weight of deacetylated chitin in one hundred parts by volume of two percent acetic acid with efficient stirring until most of the solid has dissolved. The insoluble residue is removed by centrifugation and decantation and the resulting solution, which may be gelatinous, is neutralized with sodium hydroxide, e. g. 2.5 N, using universal indicator strip paper to determine the end-point. A white precipitate is formed and collected, as by centrifugation, and may be washed, as with the successive use of distilled water, alcohol, absolute alcohol, ether and dry pyridine. This precipitate many finally be suspended in dry pyridine as a pale brown colloid. The volume of pyridine in milliliters may be about eight times the original weight in grams of starting material. The purity of pyridine used may be critical in some cases. Thus, one sulfation experiment using redistilled pyridine boiling over a three degree range produced a satisfactory yield and the product exhibited anti-coagulant activity of the order of 30 Roche anti-coagulant units per milligram.

The activation procedure discussed above, in addition to any purification which it might render, produced a colloidal suspension which was readily susceptible to sulfation. Other procedures for the formation of colloidal suspensions of the material to be sulfated will be apparent to those skilled in the art, e. g. mechanical reduction of particle size as by grinding or the production of particles from individual molecules in solutions, as by controlled precipitation.

The reagents used for sulfation include chlorosulfonic acid, e. g. 10 ml. dissolved slowly in the cold in six volumes of freshly distilled, dry pyridine when it is desired to use pyridine as a solvent. Sulfation with this reagent may be carried out by heating for a short time, as for one hour on the steam bath after the above solution of chlorosulfonic acid in pyridine has been mixed with activated deacetylated chitin (about 3.5 grams) in about 40 ml. of pyridine.

The crude reaction product may be recovered by pouring the mixture into water, neutralizing with alkali and precipitating the crude sulfated deacetylated chitin, in the form of its sodium salt when the alkali is sodium hydroxide, by the addition of a water-soluble organic solvent such as ethanol.

Another suitable reagent for sulfation is sulfur trioxide. Thus, four equivalents of sulfur trioxide (freshly distilled monomer) per nitrogen may be used in liquid sulfur dioxide e. g. 20 cc. per gram of N-deacetylated chitin, to sulfate the activated starting reagent. Use may be made of mixed solvents, including alcohol-free chloroform. The reaction can be carried out slowly at lower temperatures, such as 30 hours at —20° C., and it will be obviously advantageous to use a dry, closed pressure system. Removal of the solvent under reduced pressure leaves a solid product which may be neutralized with alkali, such as sodium hydroxide, and in that case precipitated with ethanol as an amorphous, water-soluble solid.

Further purification of the products of this invention may be desirable and in particular may remove traces of toxic materials or may increase the anti-coagulant potency of the product. Thus careful centrifugation and subsequent fractional precipitation as from alcohol and water may be used to give a purified product. Alternately or in addition, use may be made of dialysis by dissolving the product in water, dialyzing for about two days against running water to remove impurities, e. g. inorganic sulfate, concentrating in vacuo, adding a solution of saturated sodium chloride to the concentrate and precipitating the product as its sodium salt by the addition of selected water-soluble organic solvents, such as alcohols. The salt may be washed with organic solvents, such as alcohol and ether, and may be dried in vacuo in the presence of drying agents such as phosphorous pentoxide.

The products may be obtained as amorphous powders ranging in color from cream to brown. After sufficient purification they will be found to dissolve readily in water to form a clear solution in which no free inorganic sulfate is detectable. The solutions rotate the plane of polarized light. The products, when properly prepared with extensive sulfation of free amino groups and without excessive depolymerization or degradation, will show anticoagulant activities of the order of magnitude of 30 or 250 Roche units per mgm. when tested by a modified Kuizenga method using citrated sheep plasma. These figures are given as examples only, however, and are not to be considered as definitive or constituting the limits of a range of activity. In particular cases, the values obtained will differ markedly depending on factors mentioned previously. When carefully prepared, the products give a negative ninhydrin test for free amino groups.

The number of sulfate groups attached to each disaccharide unit, where the polymeric carbohydrate may be considered to be composed of such repeating units, will vary with the number of hydroxyl and amino groups originally present, with the degree to which deacetylation of nitrogen and oxygen was effected and with the extent of sulfation. Control of these factors can be exercised by those skilled in the art by choice of starting materials and by proper selection from the processes and procedures described above.

Thus in one preparation from chitin, the product, which assayed 250 Roche units per milligram and had

$$(\alpha)_D^{28} = -23$$

in water gave the following analysis:

Found:	Percent
C	20.54
H	2.95
N	3.41
S	15.8
Na	11.2

It may be theorized that these results are in fair agreement with an empirical formula of $C_{12}H_{19}O_{17}N_2S_3Na_3$ and that in this case there are three sulfate units for each disaccharide unit. It may further be theorized that the nitrogen groups may be considered to be sulfated, leaving one sulfate acid ester per disaccharide unit, since the starting material, N-deacetylated chitin, is thought on theoretical grounds to contain two amino groups and four hydroxyl groups available for sulfation per each disaccharide unit and since the product exhibited a negative ninhydrin test. The degree of sulfation in this product was slightly higher than that of heparin, which is thought to carry five sulfate units per tetrasaccharide unit. It is also theorized that this product contained the same type of nitrogen-to-sulfur linkage as heparin on the basis of their similar behaviour in the Van Slyke amino assay wherein the percentage total nitrogen increased at a rate which decreased with time.

In another preparation the product had a sulfate content of 15.4% which is thought to correspond to about three sulfate groups per disaccharide unit.

It will be understood that, without departing from the spirit of the invention or the scope of the claims, various modifications may be made in the specific expedients described. The latter are illustrative only and not offered in a restricting sense.

I claim:

1. A member of the class consisting of N-deacetylated chitin sulfated on nitrogen and non-toxic organic and inorganic salts thereof.

2. A member of the class consisting of N-deacetylated chondroitin sulfated on nitrogen and non-toxic organic and inorganic salts thereof.

3. A member of the class consisting of N-deacetylated hyaluronic acid sulfated on nitrogen and non-toxic organic and inorganic salts thereof.

4. A member of the class consisting of N-deacetylated chitin bearing —$NHSO_3H$ groups attached to a plurality of carbon atoms, no more than one —$NHSO_3H$ group being attached to any one carbon atom, said carbohydrate containing substantially 15% of covalently bound sulfur, and non-toxic organic and inorganic salts thereof.

5. An alkali metal salt of N-deacetylated chitin bearing —$NHSO_3H$ groups attached to a plurality of carbon atoms, no more than one —$NHSO_3H$ group being attached to any one carbon atom, said carbohydrate containing substantially 15% of covalently bound sulfur.

6. A sodium salt of N-deacetylated chitin bearing —$NHSO_3H$ groups attached to a plurality of carbon atoms, no more than one —$NHSO_3H$ group being attached to any one carbon atom, said carbohydrate containing substantially 15% of covalently bound sulfur.

7. A compound selected from the group consisting of (*a*) a member of the class consisting of N-deacetylated chitin sulfated on nitrogen, N-deacetylated chondroitin sulfated on nitrogen and N-deacetylated hyaluronic acid sulfated on nitrogen, and (*b*) non-toxic organic and inorganic salts thereof.

8. A compound having anti-coagulant activity consisting of N-deacetylated chitin sulfated on nitrogen.

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