Summary

In order to obtain the heparin-like active substances, the sulfates of oxidized starch, obtained by oxidation with nitric acid and its derivatives, were prepared by the reaction with formamide and chlorosulfonic acid. Sulfate of reduced product of oxidized starch, sulfates of oxidized starch amine, sulfates of methyl ester of reduced product of oxidized starch and its reduced product, and demethyl product were also prepared. Reduced product of oxidized starch is found more suitable as a starting material to prepare the heparin-like substances, because reduced product of oxidized starch having no carbonyl group is more stable against degradation during sulfation.

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27. Masaya Namekata and Sachiko Iwai: Studies on Oxidized Starch Sulfates for Medical Purposes. II.[1] Anticoagulant Activity of Sulfates of Oxidized Starch and its Derivatives.

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It is well known that polysaccharide polysulfates have a heparin-like anticoagulant activity and studies on several synthetic heparinoids have been made.[2~6] In the preceding paper,[1] the synthesis of sulfates of oxidized starch and its derivatives was reported. In the present work, the anticoagulant activity of these compounds and its relationship to the sulfur content and/or to intrinsic viscosity were examined.

Experimental

Materials

a) Heparin sodium: Japanese Pharmacopoeia Standard 108 U./mg. distributed by National Hygienic Laboratory.
b) Dextran sulfate: British Pharmacopoeia Standard distributed by the Byron Chemical Co.
c) Sulfates of oxidized starch and its reduced product.[1]
d) Sulfated whole blood: 250 cc. of bovine blood was collected in a wide-necked, glass-stoppered bottle containing 50 cc. of a 10% (w/v) solution of anhyd. Na_2SO_4 in H_2O, immediately after slaughtering and stored below 4° until use.
e) Acetone-dried bovine brain: A fresh bovine brain previously freed from vascular and connective tissues was cut into small pieces and placed in Me_2CO for preliminary dehydration. In order to complete the dehydration, 30 g. of the residue was pounded in a mortar repeatedly with the addition of 75 cc. of Me_2CO and filtered. Finally it was dried over P_2O_5 at room temperature in vacuum.
f) Thrombokinase extract: 2 g. of acetone-dried bovine brain was extracted with 40 cc. H_2O for 15 min. at 45°, centrifuged for 10 min. at 2500 r.p.m., and filtered. The filtrate was stored at below 4°

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until use. 1) Determination of the sulfur content and intrinsic viscosity were made as previously reported.[1] 2) Determination of anticoagulant activity: Evaluation of anticoagulant activity was performed according to the assay of heparin in the Japanese National Formulary II, as referred to the Bulletin of National Hygienic Laboratory.[7] Standard heparin sodium (J. P.) was used as the standard. Three dilutions of standard preparation containing 1.28, 1.6, and 2.0 units per cc. of water and three expected equivalent dilutions were prepared in 150×13 mm. test tubes and 0.2 cc. of the thrombokinase extract was added; the concentration of the thrombokinase extract was so selected that the longest clotting time may range from 9 to 12 min. 1.0 cc. of sulfated whole blood was added and mixed by gentle inversion. For each tube, the time was recorded to the nearest 15 sec. from this addition to the formation of a firm clot which remained in the bottom of the tube, when it is completely inverted. The comparison was repeated 4 times for a complete assay. The result was calculated by the standard statistical method.
3) Stability of heparinoids against acid or alkali: Test sample was dissolved in 1 cc. each. of 1/6*N* HCl, 1/6*N* NaOH and 1/6*N* NaCl, and kept for 24 hr. at room temperature (or 3 hr. at 50°). Then it was neutralized with HCl or $NaHCO_3$ (pH ca. 5.) and diluted to the adequate concentration for the anticoagulant assay. Anticoagulant activity was determined and recovery rate was calculated in percentage.

Results and Discussion

First, the effect of temperature on coagulation time was examined. As shown in Fig. 1 the time of coagulation is shortened with elevation of temperature. Therefore, the determination of anticoagulant activity was carried out at 20°.

Log Dose-response curves of heparin (J. P. standard), dextran sulfate (B. P. standard), and oxidized starch sulfate are illustrated in Fig. 2, where, at the range between 1.28 and 2.0 U, the three curves are respectively linear and parallel, but in units lower than 1.2 U, the oxidized starch sulfate curve rises over that of heparin. This result is in accord with Forwell's report on dextran sulfate.[8]

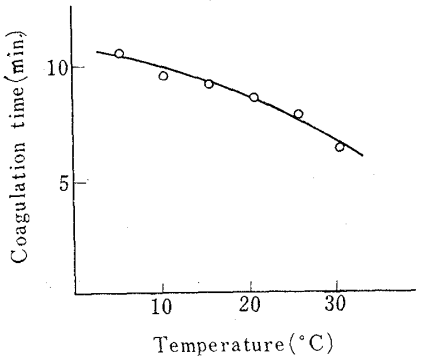

Fig. 1. Effect of Temperature on the Coagulation Time

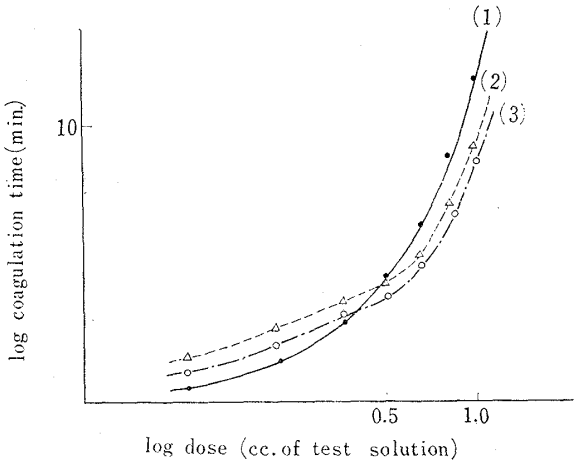

Fig. 2. log Dose-Response Curves of Heparin Standard (J. P.), Dextran Sulfate (B. P.), and Oxidized Starch Sulfate
(1) Heparin 1.85 mg./cc. = 2.0 unit. (2) Dextran Sulfate 84 mg./cc.
(3) Oxidized Starch Sulfate 3.42 mg./cc.

With the factorial analysis of these data on the parallelism over the range between 1.28 and 2.0 units, three lines are statistically proved as being linear and parallel ($p < 0.05$). Fiducial limits of error, according to Finney's formulae (*g*-criterion), is calculated as 98% and 102%.

7) K. Nagasawa, G. Nakayama, J. Serizawa: Eisei Shikenjo Hokoku, **75**, 207 (1957).
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Table I. Anticoagulant Activity and Analytical Data of Sulfates of Oxidied Starch and its Derivatives

Compound No.*	Sulfur content (%)	Sulfur content (mole)	Carboxyl content (%)	Carboxyl content (mole)	Viscosity [η]	Anticoagulant activity U/mg.
ROS18-S. 1	15.73	1.75	7.70	0.41	0.040	27.7
OS12-S. 3	15.33	1.68	8.12	0.42	0.010	6.2
OS17-S. 1	14.16	1.54	9.70	0.49	0.009	5.5
ROS17-S. 1	15.30	1.67	9.31	0.49	0.038	35.8
OS.Y-S. 1	11.81	1.04	8.92	0.38	0.011	16.7
ROS.Y-S. 1	16.38	1.89	7.73	0.43	0.036	42.4
OS24-S. 1	11.97	1.09	11.81	0.52	0.006	0.1
ROS24-S. 1	15.93	1.79	7.80	0.42	0.038	33.2
ROS26-S. 1	3.83	0.25	17.89	0.55	0.015	0.3
ROS26-S. 2	11.19	0.99	13.45	0.57	0.015	7.2
ROS26-S. 3	12.47	1.18	12.20	0.55	0.015	9.7
ROS26-S. 4	13.80	1.59	11.01	0.57	0.015	11.3
ROS26. F1-S	14.22	1.54	14.43	0.75	0.010	8.6
ROS26. F2-S	14.60	1.65	15.58	0.84	0.009	5.5
ROS26. F3-S	14.36	1.53	13.61	0.62	0.008	4.2
ROS26. F4-S	16.00	1.84	8.94	0.49	0.006	2.2
OS26-S. 2	9.66	0.80	14.24	0.56	0.007	0.4
OS26-S. 5	11.21	1.00	14.31	0.61	0.007	0.6
OS26-S. 9	9.86	0.82	14.41	0.58	0.006	0.1
OS27-S. 2	12.24	1.08	15.60	0.69	0.007	2.0
OS27-S. 4	12.70	1.21	11.37	0.52	0.007	3.2
OS27-S. 5	12.00	1.16	16.57	0.76	0.007	1.6
ROS27-S. 1	12.71	1.23	13.67	0.63	0.011	8.1
ROS27-S. 2	13.72	1.41	12.27	0.60	0.011	11.0
ROS27-S. 3	13.88	1.44	12.93	0.64	0.011	14.8
ROS26. R'-S	15.11	1.61	7.27	0.37	0.011	8.4
ROS26. Me-S	13.56				0.011	2.5
ROS26. H-S	13.67	1.49	17.00	0.88	0.011	3.4
ROS21-S. 1	15.53	1.70	7.70	0.40	0.012	8.4
OS. NH_2-S	10.94		12.05		0.005	0.1
OA-S	11.13	0.93	8.26	0.33	0.020	8.8
OAP-S	10.72	0.89	9.68	0.39	0.023	9.3
Dextran sulfate	15.3					29.5

* OS (oxidized starch), ROS (reduced product of oxidized starch), OA (oxidized amylose), OAP (oxidized amylopectine), -S (sulfate), F1 (fraction 1), NH_2 (amine), R' (reduced product of metylester), Me (methyl ester), H (demethyl product of methyl ester).

Results on anticoagulant activity, sulfur content, and intrinsic viscosity of synthetic heparinoids are listed in Table I. In the series of compound No. ROS26-S. 1 to S. 4, having a similar level of intrinsic viscosity but different sulfur content, anticoagulant activity gradually increased with the increasing sulfur content. Also, in the series of compound No. ROS 26. F1-S to F4-S, having a similar value of sulfur content but different viscosity or molecular size, anticoagulant activity increased gradually with the increasing molecular size.

On the other hand, the compounds of very small molecular size with relatively high sulfur content and the compounds of very low sulfur content with relatively large molecular size show very weak anticoagulant activity. These results suggest the necessity of a certain level of sulfur content and of molecular size for the activity. If so, the weak activity of aminopolysaccharide sulfates could be explained by its small molecular size due to the degradation of the molecule in the course of amination.

The analysis of multiple correlation among anticoagulant activity, sulfur content, and viscosity on 17 samples from the data in Table I is summarized as follows :

1) The multiple correlation coefficients among the three were highly significant by F-

test ($p<0.005$). 2) The partial correlation coefficient (r) of viscosity against anticoagulant activity at a fixed sulfur content, and the coefficient of sulfur content against anticoagulant activity at a fixed viscosity, were calculated respectively as 0.868 and 0.762 ($p<0.01$), but the coefficient of viscosity against sulfur content at a fixed anticoagulant activity is -0.611 ($p<0.05$). The reversed correlation between viscosity and sulfur content could be explained as due to the condition of esterification, because introduction of an increasing amount of sulfur may promote degradation of the polysaccharide molecule. This fact indicates that viscosity (molecular size) seems to be a more important factor for appearance of anticoagulant activity.

Toxicity of these compounds are shown in Table II. The compounds of high viscosity show strong toxicity, as reported by Ricketts.[9]

Table II. Toxicity (LD_{50}) of Sulfates of Oxidized Starch and its Derivatives

Compound No.	LD_{50} (mg./kg.)	Range p 0.05	Sulfur content		Carboxyl content		Viscosity [η]	Anticoagulant activity U/mg.
			(%)	(mole)	(%)	(mole)		
OS26-S. 9	1689	(1430~1990)	9.86	0.82	14.41	0.58	0.006	0.1
ROS18-S. 1	63	(57~69)	15.73	1.75	7.70	0.41	0.040	27.7
OS12-S. 3	1287	(1120~1490)	15.33	1.68	8.12	0.42	0.010	6.2
OS.Y-S. 1	694	(590~820)	11.81	1.04	8.92	0.38	0.012	16.7
ROS26. Me-S	1315	(1014~1706)	14.56				0.012	2.5
ROS26. R′-S	1069	(803~1424)	15.11	1.61	7.27	0.37	0.011	8.4
ROS26. H-S	1541	(1262~1883)	13.67	1.49	17.00	0.88	0.011	3.4
OA-S	1296	(1081~1554)	11.13	0.93	8.26	0.33	0.020	8.8
OAP-S	1379	(1160~1638)	10.72	0.89	9.68	0.39	0.023	9.3

LD_{50}: Obtained by probit method, i.v. injection for ddN mice.

In order to examine the effect of carboxyl residues upon the toxicity, as reported by Karrer, *et al.*,[2] three derivatives, compound No. ROS26. Me-S, compound No. ROS26. R′-S and compound No. ROS26. H-S, were compared, and the toxicity decreased in the order of compound No. ROS26. H-S, compound No. ROS26. Me-S and compound No. ROS26. R′-S in accord with Karrer's results,[2] in which the compounds with carboxyl residues have a weaker toxicity. Oxidized starch sulfate, sulfates of reduced product of oxidized starch, and heparin were compared for their stability against acids and alkalis. Table III shows that sulfates of reduced product of oxidized starch is more stable than oxidized starch sulfate and heparin is the most unstable at 50° against HCl. It is suggested that C-6 residue in oxidized starch sulfate could be the cause of its instability in alkaline solution, while N-sulfate in heparin could be the cause of its instability against acid.

Table III. Stability of Heparin, Sulfates of Oxidized Starch, and its Derivatives against Acids and Alkalis

		Recovery %		
		Heparin	ROS27-S. 2	OS27-4
Room temp. 24 hr. (25~30°)	1/6N NaOH	100	100	85
	1/6N HCl	100	100	92
	1/6N NaCl	100	100	100
50° 3 hr.	1/6N NaOH	93	82	86
	1/6N HCl	37	55	56
	1/6N NaCl	96	100	100

In conclusion, results of the present series of experiments coincide with that of polysaccharide sulfates, reported by many workers, and it can probably be expected that toxicity of oxidized starch sulfate containing a carboxyl residue at C-6 in the glucose

9) C. R. Ricketts: Biochem. J., 51, 129 (1952).

unit is less than that of other polysaccharide sulfates which does not contain any carboxyl residue.

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Summary

Anticoagulant activity of synthetic heparinoids was determined and its relationship to the sulfur content and/or to the molecular size (viscosity) was investigated. Carboxyl residue at C-6 in the glucose unit was proved to weaken the toxicity. Heparin, oxidized starch sulfate and sulfate of the reduced product of oxidized starch were compared for their stability against acids and alkalis.

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28. **Masaya Namekata**: Studies on Oxidized Starch Sulfates for Medical Purposes. III.*1 Inhibition of the Proteolytic Action of Pepsin by Sulfates of Oxidized Starch and its Reduced Products.

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It is known that sulfate-containing polysaccharides inhibit the proteolytic action of pepsin. The inhibitory action of natural or semi-synthetic polysaccharide sulfates, such as chondroitin sulfuric acid, heparin, polyhydromannuronic acid sulfate, and amylose sulfate upon proteolysis has been reported,[1~4] but these were limited to comparative studies on the inhibitory action of polysaccharides.

In the previous reports of this series from this Laboratory, the preparation of oxidized starch, its reduced products and their respective sulfates and their blood anticoagulant activities were reported. In the present paper, the inhibitory action of oxidized starch sulfate and sulfate of reduced product of oxidized starch on the proteolytic action of pepsin is reported.

Experimental

Determination of Proteolytic Action of Pepsin——The proteolytic action of pepsin was determined by the method of Bonfil, *et al.*[3,5] 1 cc. of crystalline pepsin solution (1 mg./cc.), adjusted to pH 1.6 by addition of conc. HCl, was placed in a test tube, then either 1 cc. of a solution of polysaccharide sulfates or 1 cc. of distilled water (both were adjusted to pH 1.6 by conc. HCl) was added,

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