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26. Yoshihiro Nitta, Masaya Namekata, Eiichi Tomita, and Yoshiharu Nawata: Studies on Oxidized Starch Sulfates for Medical Purposes. I.[*1] Synthesis of Sulfates of Oxidized Starch and its Derivatives.

(*Research Laboratory, Chugai Pharmaceutical Co., Ltd.*[*2])

It is known that heparin is a polysaccharide sulfate, composed of glucuronic acid and glucosamine (1:1), and the sulfate groups in its molecule are necessary for its biological activity.[1~5] Many polysaccharide sulfates, starting from starch,[6,9] dextran,[7] alginic acid,[8] and others[9] have been studied as the heparin-like substances. In this laboratory, as one of the research program on utilization of oxidized starch, which is composed mainly of glucuronic acid, oxidized starch and its derivatives were sulfated and their heparin-like activity such as blood anticoagulation, inhibition of proteolytic action of pepsin, and lipemia clearance were examined. In this paper, the details on the synthesis of the sulfates of oxidized starch and its derivatives are reported.

I. Preparation of Oxidized Starch and its Derivatives

i) **Oxidation of starch**——It is known that blood anticoagulant activity and toxicity of polysaccharide sulfates depend on the $\overline{\mathrm{DP}}$ (degree of polymerization) and molecular structure of the starting materials. First, the oxidation of potato starch with nitrogen dioxide[10,11] (I), nitric acid[12] (Ⅱ), and by the method (Ⅲ) or a modified method[13] of (Ⅱ), were examined in order to obtain the products with various $\overline{\mathrm{DP}}$ and carboxyl group content. The products, isolated from the reaction mixture with MeOH, were analyzed for $\overline{\mathrm{DP}}$ by viscometry and for carboxyl groups by conductometric titration to know the

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[*2] Takadaminami-cho, Toshima-ku, Tokyo (新田義博, 行方正也, 富田栄一, 縄田喜治).

1) E. Jorpes: Biochem. J., **36**, 203 (1942).
2) E. Jorpes, B. Strom: J. Biol. Chem., **118**, 447 (1937),
3) M.L. Wolfrom, R. Montgomery, *et al.*: J. Am. Chem. Soc., **72**, 5796 (1950).
4) A.B. Foster, A.J. Huggard: "Advances in Carbohydrate Chemistry," Ed. by M.L. Wolfrom, Vol. **10**, 355 (1953), Academic Press Inc., New York.
5) L.B. Jaques, H.J. Bell: "Method of Biochemical Analysis," Ed. by David Glick, Vol. **7**, 253 (1959), Interscience Publishers, Inc., New York.
6) T. Astrup, J. Piper: Acta Physiol. Scand., **11**, 211 (1946).
7) C.R. Rickets: Biochem. J., **51**, 129 (1952); Chem. & Ind. (London), **1952**, 869.
8) J. Seifter, A.J. Begany: Am. J. Med. Sci., **216**, 234 (1948).
9) E. Chargraff, F.W. Bancraft, S. Brown: J. Biol. Chem., **115**, 155 (1936).
10) W.O. Kenyon, E.C. Yachel: J. Am. Chem. Soc., **64**, 121 (1942).
11) W.O. Kenyon, *et al.*: U.S. Pat. 2,472,590 (1949).
12) K. Heyns, G. Graefe: Chem. Ber., **86**, 646 (1953); D.P. 849,642 (1951).
13) Y. Nitta, Y. Nakajima, A. Momose, K. Ando: Japan. Pat. 241,792 (1947).

extent of oxidation. It was found that starch underwent heavy oxidative degradation with procedure (Ⅱ) and (Ⅲ), while a slight degradation with procedure (I), as the intrinsic viscosity of (Ⅱ) and (Ⅲ) are much smaller than that of (I). It was also found that the difference in $\overline{DP}$ of the product from (I) to the others is rather large. Starch was oxidized in a short time by the method (Ⅱ), in order to obtain products with viscosity between those obtained by (I) and (Ⅱ). The products which have various carboxyl content and suitable viscosity for sulfation were obtained. Amylose and amylopectin were oxidized with nitric acid, and the oxidized products were respectively obtained. In Table I, the properties of the products are shown.

TABLE I. Analytical and Other Data on Oxidized Starch, Reduced Product of Oxidized Starch, Oxidized Amylose, and Oxidized Amylopectin

Compound No.	COOH (%)	$[\eta]$	Oxidation conditions		Reagent
			Temp. (°C)	Time (hr.)	
OS- 9	22.0	0.012	30	5	60% HNO_3
OS-10	25.2	0.023	30	5	60% HNO_3
OS-12	22.9	0.225	0～10	72	HNO_3 (d 1.42)
ROS-12	20.4	0.060	Reduced product of No. OS-12		
OS-15	22.0	0.662	0～10	168	$N_2O_4 \cdot CCl_4$
OS-16	21.7	0.714	0～10	168	$N_2O_4 \cdot CCl_4$
OS-17	19.27	0.357	0～10	72	HNO_3 (d 1.42)
OS-18	5.02	0.894	0～10	48	HNO_3 (d 1.42)
ROS-18	4.88	0.811	Reduced product of No. OS-18		
OS-22	29.30	0.240	0～10	92	HNO_3 (d 1.42)
ROS-22	17.05	0.157	Reduced product of No. OS-22		
OS-24	26.85	0.095	0～10	168	HNO_3 (d 1.42)
OS-26	18.11	0.123	0～ 5	168	HNO_3 (d 1.42)
ROS-26	17.13	0.090	Reduced product of No. OS-26		
OS-27	19.41	0.191	0～ 5	168	HNO_3 (d 1.42)
ROS-27	11.80	0.110	Reduced product of No. OS-27		
OS-Y	14.01	0.414	0～ 5	168	HNO_3 (d 1.42)
ROS-Y	8.72	0.326	Reduced product of No. OS-Y		
OA	28.06	0.055	0～10	168	HNO_3 (d 1.42)
OAP	27.58	0.073	0～10	168	HNO_3 (d 1.42)

* OS (oxidized starch), ROS (reduced product of oxidized starch),
OA (oxidized amylose), OAP (oxidized amylopectin).

ii) **Preparation of Reduced Product of Oxidized Starch**——These oxidized starches are not only pure polymers of glucuronic acid, but also contain carbonyl groups in the molecule, due to partial oxidation of the secondary alcohol at C-2 and C-3 by a side reaction during the course of oxidation of primary alcohol in the starch. These facts have been proved by Imai, *et al.*[14~17] who reported that oxidized strarch was more easily degraded both in acid and alkaline solution than reduced product of oxidized starch. Reduced product of oxidized starch was obtained by treating oxidized starch with sodium borohydride in ice bath according to the method of Imai, *et al.*[16] In Table I, the physicochemical properties of reduced product of oxidized starch are also shown, for comparison with those of oxidized starch. It is indicated that reduced product of oxidized starch has smaller value of viscosity and carboxyl content than those of oxidized starch. This low viscosity of reduced product of oxidized starch would be caused by the degradation of oxidized starch at the beginning of reduction with sodium borohydride, and high carboxyl value of oxidized starch (a little higher than theoretical maximum value, in some cases)

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14) Y. Imai : Yakugaku Zasshi, **81**, 1110 (1961).
15) *Idem* : *Ibid.*, **81**, 1116 (1961).
16) Y. Imai, Y. Hirasaka : *Ibid.*, **81**, 1362 (1961).
17) Y. Imai, S. Owari : *Ibid.*, **81**, 1221 (1961).

may be due to the formation of the nonuronic acid groups by alkaline degradation of oxidized starch during the analysis by alkaline titration. These results coincide with those of Imai, *et al.*[14~16]

iii) Preparation of Oxidized Starch Amines——Wolfrom, *et al.*[18] reported that a part of sulfate groups in heparin was combined with amino groups and anticoagulant activity was affected more by N-sulfate groups than the O-sulfate groups. Oxidized starch amine is prepared by catalytic reduction of the carbonyl groups of oxidized starch in ammoniacal methanol solution, by the modified procedure for preparation of glucamine from glucose.[19] Nitrogen content of oxidized starch amine was 5.38%. The presence of amino groups in this product was proved by infrared absorption spectrum and also by paper chromatography. There was a strong band near 1600 cm^{-1}, which might be assigned to COO^- or NH_3^+ (Fig. 1). Paper chromatographic examination of hydrolysate of oxidized starch amine with dilute hydrochloric acid showed the presence of a reducing sugar by reduction of silver nitrate and of amino sugar by Ninhydrin and the Ehrlich reagent (Fig. 2). The presence of glucosamine in the hydrolysate was not confirmed. A further

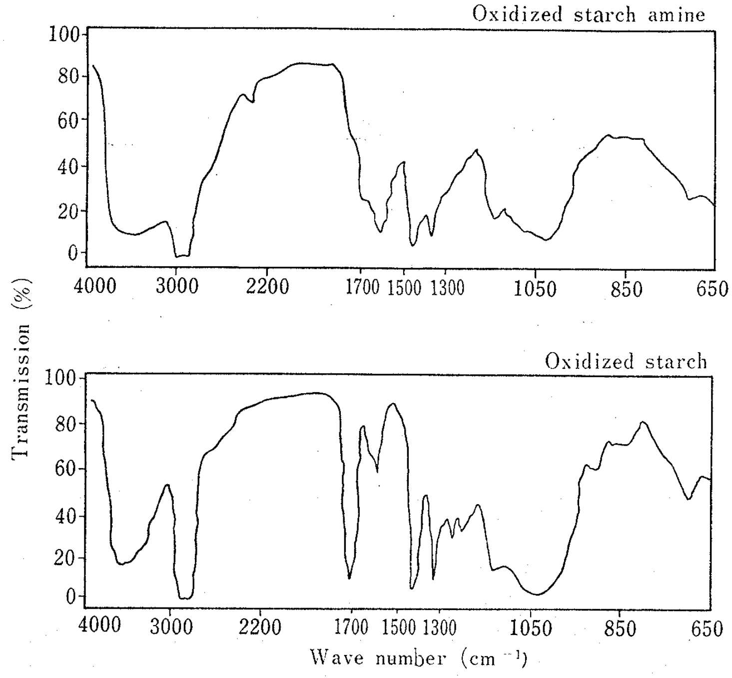

Fig. 1. Infrared Absorption Spectra of Oxidized Starch and Oxidized Starch Amine (in Nujol mull)

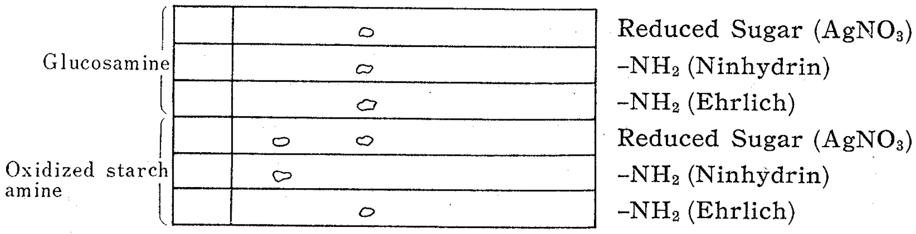

Fig. 2. Paper Chromatogram of Acid Hydrolysate of Oxidized Starch Amine
Solvent System : n-BuOH-pyridine-H_2O=6:4:3
Filter paper : Toyo Filter Paper No. 51

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[18] M.L. Wolfrom, J.M. Shan, C.G. Summers : J. Am. Chem. Soc., **75**, 1519 (1953).
[19] H. Wiston Wayne, H. Adkins : *Ibid.*, **62**, 3314 (1940).

investigation on the catalytic reduction indicated that, as was expected, no oxidized starch amine was formed from reduced product of oxidized starch by the same procedure, indicating that reduced product of oxidized starch has no carbonyl groups.

iv) Preparation of the Derivatives of Reduced Product of Oxidized Starch Methyl Ester, its Demethyl Derivative, and its Reduced Derivative——Karrer, *et al.*[20] reported on the toxicity of polysaccharide sulfates and stated that chondroitinsulfuric acid, pectin sulfate, and heparin which had acidic groups in the molecule bonded directly with carbon atom of glucose, had low toxicity while sulfates of cellulose, starch, and glycogen, which were decomposed *in vivo* forming primary alcohol at C-6, had a rather high toxicity.

Reduced product of oxidized starch (I) with the same $\overline{\mathrm{DP}}$ but various carboxyl group contents were prepared. First, methyl ester of reduced product of oxidized starch (II) was prepared by the reaction of reduced product of oxidized starch (I) silver salt with methyl iodide or directly by the reaction of (I) with methanol containing 2.5% hydrochloric acid. Reduced product of (II), (III), was prepared by the reduction of (II) with sodium borohydride in ice bath. Demethyl product of (II), or (IV), was prepared by the reaction of (II) with sodium hydroxide (Chart 1). Infrared spectra indicated the presence of methyl ester in (II) (OMe : 10.87%) ($\nu_{C=O}$ 1738 cm^{-1}) and its absence in (III). Carboxyl values for (I), (IV), and (III) were 17.08%, 17.00%, 7.28% respectively by conductometric titration. Decrease of carboxyl groups in (III) was apparent. From these analytical data, the formation of (II), and the formation of (IV) and (III) through (II) was confirmed.

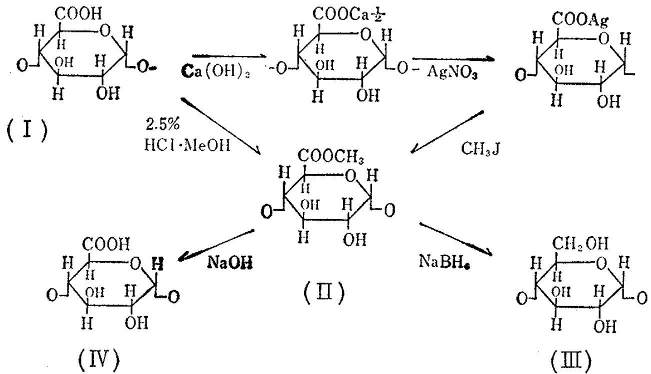

Chart 1.

There should be no serious depolymerization reaction in the formation of (II) from silver salt of (I) as the reaction temperature was kept below room temperature, but there was a considerable depolymerization by the direct reaction of (I) with MeOH containing HCl. Methylation by the latter procedure was accompanied with hydrolysis and degraded products were obtained. Reduced product of oxidized starch differing in $\overline{\mathrm{DP}}$ was obtained from the same material by this procedure.

II. Synthesis of Sulfates of Oxidized Starch and its Derivatives

A mixture of pyridine and chlorosulfonic acid[4] has been generally used as a sulfating agent for polysaccharides. In re-examination of the sulfation with pyridine-chlorosulfonic acid mixture, good results were not obtained, because oxidized starch was hydrolyzed during the sulfation, probably owing to the carbonyl groups in its molecule. The sulfation procedure was changed to the formamide-chlorosulfonic acid method, in which reaction could be carried out at room temperature or below, and oxidized starch sulfates, not hydrolyzed to a great extent during sulfation, were obtained in a better yield.

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[20] P. Karrer, J. Koenig, E. Usteri : Helv. Chim. Acta, **26**, 1296 (1943).

The condition of sulfating oxidized starch with formamide and chlorosulfonic acid mixture was examined at 20°, 40°, and 60°, varying reaction time to 3, 6, 12, and 24 hours at each temperature. The products were analyzed for sulfur content, carboxyl value, and intrinsic viscosity. The relationship between these conditions (temperature and time) and analytical data on products (S, COOH, $[\eta]$) are shown in Table II.

Table II. Relationship between the Condition of Sulfation and Products

Reaction conditions		Sulfur content		Carboxyl content		$[\eta]$
Temp. (°C)	Time (hr.)	%	mole	%	mole	
20	3	7.40	0.55	15.65	0.56	0.0078
	6	9.66	0.80	14.24	0.56	0.0074
	12	10.71	0.94	14.50	0.61	0.0064
	24	10.93	0.97	14.49	0.61	0.0064
40	3	11.21	1.00	14.31	0.61	0.0072
	6	11.07	0.97	13.13	0.55	0.0072
	12	10.71	0.94	15.22	0.67	0.0064
	24	10.66	0.94	15.28	0.66	0.0062
60	3	9.86	0.82	14.41	0.58	0.0062
	6	9.54	0.77	13.55	0.53	0.0060
	12	9.71	0.73	14.83	0.51	0.0058
	24	9.81	0.81	13.54	0.58	0.0055

Viscosity of the product by the reaction at 20° for 24 hours decreased slightly and sulfur content increased with increasing reaction time. This means that there was a slight hydrolysis of oxidized starch during the sulfation. At 40° and 60°, reaction became violent, resulting in the degraded product; the viscosity and sulfur content of the products decreased with increasing reaction time. This means that oxidized strarch was degraded during sulfation at elevated temperatures owing to the carbonyl groups in molecule. The chemical stability of oxidized starch and reduced product of oxidized starch during sulfation were examined by measuring the intrinsic viscosity, sulfur content, and carboxyl value of both sulfates, which were sulfated at 40° for 6, 12, or 24 hours, respectively (Table III). It was noted that reduced product of oxidized starch was moderately esterified even at 40°, sulfur content increasing with the increase of reaction time, as at 20°, without severe degradation. On the other hand, oxidized starch was esterified, accompanying degradation, as shown in Table II. From the above facts, it can be concluded that the degradation of oxidized starch during sulfation may be caused by the presence of carbonyl groups in its molecule, and reduced product of oxidized starch is more preferable to oxidized starch, as a starting material for the synthesis of oxidized starch sulfates as the former is more stable against degradation during sulfation. Sulfates of oxidized starch and its derivatives, having various sulfur content and carboxyl value, were obtained by changing the conditions as above, with formamide

Table III. Effect of the Type of Sulfation of Oxidized Starch and Reduced Product of Oxidized Starch on Sulfur and Carboxyl Content and Intrinsic Viscosity

Materials	Reaction conditions		Sulfur content		Carboxyl content		$[\eta]$
	Temp. (°C)	Time (hr.)	%	mole	%	mole	
Oxidized starch	40	6	13.67	1.21	16.58	0.76	0.0111
		12	12.00	1.61	16.57	0.76	0.0082
($[\eta]=0.191$)		24	10.83	0.98	17.06	0.74	0.0074
Reduced product of oxidized starch	40	6	12.71	1.23	13.67	0.63	0.0108
		12	13.72	1.41	12.27	0.60	0.0107
($[\eta]=0.0110$)		24	13.88	1.44	12.93	0.64	0.0103

and chlorosulfonic acid at 20° to 25° for 24 hours. Crude products were precipitated with ethanol and redissolved in aqueous sodium carbonate solution. The sodium salts of crude product were purified, removing ammonia due to formamide in a reduced pressure, and subjecting to dialysis in a Cellophane bag against running tap water to remove inorganic ions. The solution in the Cellophane bag was concentrated in a reduced pressure and the purified product was precipitated with ethanol. Analytical and other data for sulfates of oxidized starch and its derivatives are shown in Table IV.

TABLE IV. Analytical and Other Data on Oxidized Starch and Reduced Product of Oxidized Starch and their Derivatives

Compound No.	Sulfur content		Carboxyl content		$[\eta]$
	%	mole	%	mole	
OS12-S. 3	15.33	1.68	8.4	0.42	0.010
OS17-S. 1	14.61	1.54	9.70	0.49	0.009
ROS17-S. 1	15.30	1.67	9.31	0.49	0.038
ROS18-S. 1	15.73	1.75	7.70	0.41	0.038
OS.Y-S. 1	11.81	1.04	8.92	0.38	0.011
ROS.Y-S. 1	16.38	1.89	7.73	0.43	0.036
OS24-S. 1	6.75	0.49	13.17	0.42	0.004
OS24-S. 3	11.99	1.09	11.81	0.52	0.006
ROS24-S. 1	15.98	1.79	7.80	0.42	0.038
OS26-S. 1	7.40	0.55	15.65	0.56	0.007
OS26-S. 2	9.66	0.80	14.24	0.56	0.007
OS26-S. 3	10.71	0.94	14.50	0.61	0.007
OS26-S. 5	11.21	1.00	14.31	0.61	0.007
OS26-S. 9	9.86	0.82	14.41	0.58	0.006
ROS26-S. 1	3.83	0.25	17.89	0.55	0.015
ROS26-S. 2	11.19	0.99	13.45	0.57	0.015
ROS26-S. 3	12.48	1.18	12.20	0.55	0.015
ROS26-S. 4	13.80	1.59	11.01	0.57	0.015
ROS26. F1-S	14.22	1.54	14.43	0.75	0.010
ROS26. F2-S	14.60	1.65	15.58	0.84	0.009
ROS26. F3-S	14.36	1.53	13.61	0.62	0.008
ROS26. F4-S	16.00	1.84	8.94	0.49	0.006
OS27-S. 3	12.34	1.19	16.67	0.71	0.008
ROS27-S. 1	12.71	1.23	13.67	0.63	0.011
ROS26. R′-S	15.11	1.61	7.27	0.37	0.011
ROS26. H-S	13.67	1.49	17.00	0.88	0.011
ROS26. Me-S	13.56				0.011
ROS21. Me-S	14.56				0.012
ROS21. R′-S	19.32	2.75	5.13	0.35	0.012
ROS21-S. 1	15.53	1.70	7.70	0.40	0.012
OS. NH_2-S*	10.94		12.05	0.79	0.005
OA-S	11.13	0.93	8.26	0.33	0.020
OAP-S	10.72	0.89	9.68	0.38	0.023

* N : 4.9%

Experimental

Preparation of Oxidized Starch——1) To 100 cc. of CCl_4 and 50 cc. of N_2O_4, cooled in an ice bath, 20 g. of potato starch was added and the reaction was continued for 168 hr. with stirring at 0° to 5°. N_2O_4 was removed from the reaction mixture *in vacuo*. The product was rinsed with MeOH and dried over P_2O_5 *in vacuo* to yield 18.3 g. of a substance, $[\eta]$ 0.662, COOH 0.831 mole/glucose unit.

2) To 200 cc. of HNO_3 (d=1.42), 100 g. of potato starch and 20 g. of $NaNO_2$ were added slowly and the temperature was kept at 0° to 5°. The reaction mixture, a homogeneous paste, was stirred for 168 hr. and poured into 2 L. of MeOH. The precipitate was collected and washed several times with MeOH. The purified product was dried over P_2O_5 *in vacuo* to a white powder; yield, 91 g., $[\eta]$ 0.191, COOH 0.744 mole/glucose unit.

3) To 130 cc. of 60% HNO_3, 100 g. of starch and 1 g. of $NaNO_2$ were added slowly and oxidation was continued for 5 hr. at 30° with stirring.

The syrupy reaction mixture was poured into 3 L. of MeOH, the precipitate was collected on a sintered glass filter, washed several times with MeOH, and dried over P_2O_5 *in vacuo*. Pale yellowish white powder; yield, 72 g., [η] 0.023, COOH 0.991 mole/glucose unit.

Preparation of Reduced Product of Oxidized Starch——In 100 cc. of water, 30 g. of oxidized starch was suspended with stirring and dissolved by adding $NaHCO_3$ to pH 7.0, and 5 g. of $NaBH_4$ was added slowly to the solution. The reaction mixture was stirred for 4 hr. in an ice bath, acidified with AcOH to pH 3.0 to decompose excess of $NaBH_4$, and subjected to dialysis in a Cellophane bag against running tap water until free of BO_2 ion, detected by the flame reaction. After filtration, the purified solution was concentrated *in vacuo* at below 40°. The purified product, obtained by precipitation with EtOH, was washed with MeOH and dried over P_2O_5 to a white powder. Yield, 27.5 g., [η] 0.076, COOH 0.442 mole/glucose unit.

Preparation of Oxidized Starch Amine——In 50 cc. of cooled absolute MeOH, 5 g. of dry NH_3 was absorbed, 2 g. of Raney Ni–W6 and 10 g. of oxidized starch were added, and the mixture stirred at 40° for 16 hr. in an autoclave, filled with H_2 at 50 gauge pressure. After reaction, the product was collected on a sintered glass filter and excess of NH_3 was removed from it in a reduced pressure. The crude product was redissolved in 20 cc. of water, catalyst was filtered off, and the solution was concentrated *in vacuo* at below 40°. The purified product, obtained by precipitation with absolute EtOH, was dried over P_2O_5 *in vacuo*. Yield, 8.1 g., N 5.38%, [η] 0.009.

Preparation of Methyl Ester of Reduced Product of Oxidized Starch——1) In 400 cc. of water, 40 g. of reduced product of oxidized starch was suspended, $Ca(OH)_2$ was added to the suspension, cooled in an ice bath, with stirring to neutral pH. Reduced product of oxidized starch was dissolved in the form of a Ca salt. To the solution, $AgNO_3$ solution (equal mole to Ca) was added slowly through a dropping funnel. The Ag salt of reduced product of oxidized starch precipitated with 40 cc. of EtOH, was collected, washed with 80% EtOH and absolute EtOH, and dried over P_2O_5 *in vacuo*. Yield, 56 g. In 50 cc. of water, 50 g. of Ag salt of reduced product of oxidized starch was suspended and a mixture of 50 cc. of CH_3I and 50 cc. of MeOH was added slowly through a dropping funnel with stirring. After stirring for 3 hr., AgI was separated off by centrifugation and was concentrated *in vacuo* at below 40°. The product precipitated with EtOH was collected and dried over P_2O_5 *in vacuo* to a pale yellowish white powder. Yield, 32 g., [η] 0.087, OMe 10.87%.

2) To 350 cc. of MeOH containing HCl (2.5%), 35 g. of reduced product of oxidized starch was added and the mixture was refluxed for 2 hr. with stirring. The reaction mixture was concentrated in a reduced pressure to remove HCl. The product precipitated with Me_2CO was collected, washed with Me_2CO, and dried over P_2O_5 *in vacuo* to a pale yellowish white powder. Yield, 31 g., [η] 0.062, OMe 2.94%.

Preparation of Demethyl Derivative of Methyl Ester of Reduced Product of Oxidized Starch——A solution of 10 g. of methy ester of reduced product of oxidized starch dissolved in 50 cc. of 0.6*N* NaOH, was stirred for 3 hr. at 20~25°, neutralized with AcOH, subjected to dialysis in a Cellophane bag against running tap water until free of inorganic ion, and concentrated in a reduced pressure below 40°. The purified product, obtained by precipitation with a mixture of Me_2CO and EtOH, was washed with EtOH and dried over P_2O_5 *in vacuo* to 8.4 g. of substance; [η] 0.010, COOH 0.88 mole/glucose unit.

Preparation of Reduced Product of Methyl Ester of Reduced Product of Oxidized Starch——To a solution of 10 g. of methyl ester of reduced product of oxidized starch dissolved in 100 cc. of water, 3 g. of $NaBH_4$ was added slowly with stirring and cooling in an ice bath. The reaction mixture was allowed to stand overnight and AcOH was added to decompose excess $NaBH_4$. The solution was dialyzed against running tap water in a Cellophane bag until free of inorganic salt and concentrated *in vacuo* at below 40°. The purified product was precipitated with Me_2CO. Yield, 8.0 g., [η] 0.010, COOH 0.37 mole/glucose unit.

Sulfation of Oxidized Starch——To 200 cc. of $HCONH_2$ cooled in an ice bath, 20 cc. of $ClSO_3H$ was added dropwise through a dropping funnel. To this mixture, 10 g. of oxidized starch was added and the whole was kept at 20~25° for 24 hr. with stirring, and filtered through a sintered glass filter. The syrupy product, obtained by adding 500 cc. of EtOH to the filtrate, was washed with EtOH and MeOH, and dried over P_2O_5 *in vacuo* to yield 15.2 g. of a substance.

To the crude product dissolved in 100 cc. of water, $NaHCO_3$ was added to decompose the $HCONH_2$–oxidized starch sulfate complex and NH_3 was removed *in vacuo*. The solution was dialyzed against running tap water in a Cellophane bag for 48~72 hr. until free of inorganic sulfate and concentrated *in vacuo* at below 40°. The product, precipitated with EtOH was collected, washed with EtOH, and dried over P_2O_5 *in vacuo* to a brownish yellow powder. Yield, 9.7 g. [η] 0.0068, SO_3H 1.02 mole/glucose unit, COOH 0.79 mole/glucose unit.

Sulfation of the Derivatives of Oxidized Starch——The derivatives of oxidized starch were sulfated by the same procedure as for oxidized starch sulfate as described above.

Sulfate of Reduced Product of Oxidized Starch : White powder, 11.4 g. SO_3H, 1.23 mole/glucose unit, COOH, 0.63 mole/glucose unit, $[\eta]$ 0.011.

Sulfate of Reduced Product of Methyl Ester of Reduced Product of Oxidized Starch : Pale yellowish white powder. Yield, 10.7 g. SO_3H, 2.75 mole/glucose unit, COOH, 0.35 mole/glucose unit, $[\eta]$ 0.012.

Sulfate of Demethyl Product of Methyl Ester of Reduced Product of Oxidized Starch : Pale yellowish white powder. Yield, 9.4 g. SO_3H 1.49 mole/glucose unit, COOH 0.008 mole/glucose unit, $[\eta]$ 0.011.

Sulfate of oxidized starch amine : Brown powder. Yield, 3.2 g. SO_3H 1.02 mole/glucose unit, COOH 0.79 mole/glucose unit, $[\eta]$ 0.008, N 4.9%.

The sulfate of oxidized starch amine was obtained from 5 g. of oxidized starch amine.

Intrinsic Viscosity——Viscosity was measured with a modified Ostwald viscometer at $30.00^{\circ}\pm0.05^{\circ}$. Oxidized starch was dissolved in a phosphate buffer solution$^{6)}$ (pH 6.24) and measured at concentration of 0.2∼1.0 g./100 cc. Oxidized starch sulfate was dissolved in 1*M* NaCl$^{21)}$ and measured at the concentration of 1∼8 mmole (as a monomer)/100 cc. The intrinsic viscosity, reduced viscosity at C=O in $({}^{\eta}sp/C)-C$ curve, was calculated by the minimum square method.

Sulfur Content——Sulfur was determined by the method described in British Pharmacopoeia$^{22)}$ for dextran sulfate. Sample was oxidized with $Cu(NO_3)_2$ and $KClO_3$. Sulfur was collected as benzidine sulfate and titrated with 0.02*N* NaOH (Phenol Red indicator).

Carboxyl Group Content——Carboxyl group was determined by a modified conductometric titration method of Fujita$^{23)}$ and Katsuura.$^{24)}$

About 30 mg. of oxidized starch, weighed accurately, was suspended in 15 cc. of distilled water (free of CO_2) and dissolved by adding 0.6 cc. of 2*N* NaOH. The solution was titrated with 0.2*N* H_2SO_4, with stirring.

About 30 mg. of oxidized sulfate was dissolved in 15 cc. of distilled water and 0.6 cc. of 0.2*N* H_2SO_4 was added. The solution was titrated with 0.02*N* NaOH with stirring. Carboxyl groups were calculated from the data obtained by conductometric titration curve.

Calculation of Sulfur and Carboxyl Content——Carboxyl value of oxidized starch is not constant owing to the different degree of oxidation of oxidized starch, and the sulfur and carboxyl content of the oxidized starch sulfate cannot be expressed in weight percent. In the present work molar content of sulfur and carboxyl was calculated as a monomer. Average molecular weight for a monomer $(\overline{MW})$, sulfur content (Y%) and carboxyl content (X%) can be expressed by the following equations.

$$Y=\frac{32y}{162+102y+36x}\times100 \qquad X=\frac{67x}{162+102y+36x}\times100$$

$$MW=162+102y+36x \qquad 3\geqq y\geqq0,\ \ 1\geqq x\geqq0$$

where y and x are analytical values for sulfur and carboxyl, respectively. In Fig. 3, the molar content *vs* weight percent relation of sulfur for oxidized starch sulfates, in both case of $x=0$ and $x=1$, are shown. The plots appeared inside the two curves in the present case.

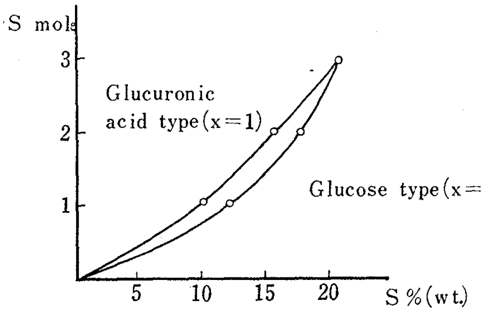

Fig. 3. Molar Content *vs.* Weight Percent Relation of Sulfur for Oxidized Starch Sulfates

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21) P. T. Mora : J. Polymer Sci., **23**, 345 (1957).
22) British Pharmacopoeia, 207 (1958).
23) H. Fujita : Memoirs of the College of Agriculture, Kyoto Univ. (1958).
24) K. Katsuura : Kôbunshi, **6**, 572 (1957).

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.

(*Research Laboratory, Chugai Pharmaceutical Co., Ltd.*[*1])

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°

[*1] Takadaminami-cho, Toshima-ku, Tokyo (行方正也, 岩井幸子).

1) Part I. Y. Nitta, M. Namekata, E. Tomita, Y. Nawata: This Bulletin, **10**, 159 (1962).

2) P. Karrer, H. Koenig, E. Usteri: Helv. Chim. Acta, **26**, 1296 (1943).

3) J. Seifter, A. J. Begany: Am. J. Med. Sci., **216**, 234 (1948).

4) H. L. Beazly, W. J. Foster, A. A. Ory, D. W. Chapman: *Ibid.*, **226**, 275 (1953).

5) D. Molho, J. Cotte: Bull. soc. chim. biol., **33**, 312 (1951).

6) C. N. Mangieri, R. Engelberg, L. O. Randall: J. Pharmacol. Exptl. Therap., **102**, 156 (1951).