of calcium and inorganic phosphate in rabbit serum. Our results are coincident with this equation (the data on calcium and inorganic phosphate levels produced by PHCP are shown in Table I).

The increases of phosphate were about three times those of calcium, but the standard errors were large in the case of inorganic phosphate, and the number of significant differences for phosphate in Table I is less than that for calcium. The concentration of inorganic phosphate in serum may be affected by more factors than that of calcium. The values of the standard errors of hydroxyproline are also larger than those of calcium. In conclusion, parotid hypocalcemic protein significantly affected the levels of calcium, inorganic phosphate, and hydroxyproline in rabbit serum.

[Chem. Pharm. Bull. 28(2) 638—641 (1980)]

Crystalline Salts of Sucrose Octasulfate

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(Received April 25, 1979)

Sulfation of sucrose with pyridine-sulfur trioxide was carried out in dimethylformamide and pyridine, and the degree of sulfation of the sodium salt of sucrose sulfate thus obtained was estimated from the ratio of sulfur to carbon content (*S*/*C*). The sulfates prepared with 9—15 molar equivalents of pyridine-sulfur trioxide in dimethylformamide and those prepared with 5 and 9 equivalents in pyridine were identified as sucrose octasulfate. Potassium, cesium, rubidium, and ammonium salts of sucrose octasulfate were obtained as crystals.

Keywords——sulfation; pyridine-sulfur trioxide; sucrose; crystalline sucrose octasulfate; sucrose sulfate

Namekata and his co-workers[2,3] prepared sodium salts of disaccharide sulfates having strong anti-pepsin and anti-ulcer activities, and a basic aluminium salt of sucrose sulfate[3] having more potent activities.

In their previous work,[2,3] the amorphous sodium salt of sucrose sulfate obtained by sulfation of sucrose using pyridine-sulfur trioxide complex[4] in pyridine was analyzed only for sulfur content, giving a rather inappropriate value due to the presence of bound water, and the structures of the prepared sulfates were not satisfactorily confirmed.

Although Takiura and his co-workers[5] reported successful separation of sucrose di- and tri-sulfates from the products obtained by sulfation of sucrose using the same agent in dimethylformamide (DMF), no other components were examined. We prepared the salts of sucrose sulfate by using various amounts of pyridine-SO_3 and attempted to estimate their degrees of sulfation.

This paper describes a method for estimating the degree of sulfation in the sucrose molecule and reports the preparation of crystalline salts of sucrose octasulfate.

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2) M. Namekata, A. Matsuo, A. Momose, and M. Takagi, *Yakugaku Zasshi*, **87**, 376 (1967).
3) M. Namekata, T. Tanaka, N. Sakamoto, and K. Moro, *Yakugaku Zasshi*, **87**, 889 (1967).
4) J.F. Scully and E.V. Brown, *J. Org. Chem.*, **19**, 894 (1954); A.G. Lloyd, *Biochem. J.*, **75**, 478 (1960).
5) K. Takiura and S. Honda, *Yakugaku Zasshi*, **87**, 1052 (1967).

Preparation of sucrose sulfates was carried out using pyridine-SO_3 in DMF and pyridine, as in the previous reports.[2,5] The resulting pyridinium salt of sucrose sulfate was converted to the barium salt by treatment with barium hydroxide. Subsequent conversion of the barium salt to the sodium salt was carried out using Amberlite IR-120 (Na^+) resin to give a hygroscopic white powder.

Instead of analyzing the sodium salt only in terms of sulfur content and by the usual electrophoretic or chromatographic technique,[6] we attempted to determine the ratio of sulfur to carbon content (S/C), because this value is independent of the amount of bound water present in the amorphous sodium salt. The value of S/C is proportional to the number (n) of sulfate groups in a sucrose molecule; $n=4.5\times S/C$.

This was applied to the amorphous sodium salt of sucrose 1′,6′,6-trisulfate, prepared from 3′,4′,2,3,4-penta-O-acetylsucrose[7] (compound No. 8 in Table I), and gave a reasonable n value.

Table I. Analysis Data for the Sodium Salts of Sucrose Polysulfate

Comp. No.	Molar ratio of pyridine SO_3/sucrose	Solvent	Analysis (%) Found C	H	S	Na	S/C	n[b]
1	3	DMF	24.16	2.88	13.04	7.13—9.51	0.54	2.4
2	5	DMF	19.21	2.68	16.37	11.1 —11.9	0.85	3.8
3	9	DMF	12.21	1.65	21.55		1.76	7.9
4	11	DMF	12.46	1.69	21.87		1.76	7.9
5	15	DMF	12.37	1.54	21.21	15.2 —15.78	1.71	7.7
6	5	Pyridine	12.08	1.65	21.33		1.77	7.9
7	9	Pyridine	12.21	1.65	21.64		1.77	7.9
8[a]	7	Pyridine	21.87	3.29	14.09		0.64	2.9

a) Obtained by sulfation of 3′,4′,2,3,4-penta-O-acetylsucrose, followed by deacetylation.
b) $n=4.5\times S/C$

Table II. Analysis Data for the Crystalline Salts of Sucrose Octasulfate

Salt of sucrose octasulfate	mp (dec.)	Water of crystallization	Analysis (%) Calcd C	H	S	K, N	Found C	H	S	K, N	S/C
K-I[a]	169°	4	10.60	1.63	18.87	23.01	10.42	1.32	18.96	23.14	1.82
K-II[b]	230°	4					10.40	1.40	18.99	23.09	1.83
Rb[a]	149°	0	8.69	0.85	15.47		8.20		15.43		1.88
Cs[a]	144°	0	7.07	0.69	12.59		6.79		12.70		1.87
NH_4[a]	130°	2	12.48	4.36	22.21	9.70	12.20	3.95	22.25	9.42	1.82

a) Needles. b) Prisms.

As shown in Table I, the S/C values of the sodium salt prepared by sulfation in DMF increased with increasing amount of pyridine-SO_3 used, but reached a plateau at 9—15 mol of the agent. Compounds Nos. 3—5 all showed almost the same value, and no change was observed on further purification. This result suggests that all of the hydroxyl groups in the sucrose molecule were sulfated, because the n value of each compound is about 8, suggesting the product to be the sodium salt of sucrose octasulfate.

6) K. Takiura and S. Honda, *Yakugaku Zasshi*, **87**, 1256 (1967); K. Takiura, H. Yuki, S. Honda, and Y. Kojima, *ibid.*, **87**, 1262 (1967).
7) G.G. McKeown, R.S.E. Serenius, and L.D. Hayward, *Can. J. Chem.*, **35**, 28 (1957).

Sulfation of sucrose in pyridine using 5 molar equivalents gave a sodium salt (compound No. 6) having the same *S/C* value as that obtained with excess agent (compounds Nos. 3—5 and 7). Comparison of compound No. 6 with compound No. 2, (prepared by sulfation using the same molar ratio of reagent, but in DMF) indicated that in the former case, 5 molar equivalents apparently gave the same result as the use of excess reagent (*i.e.*, more than 8 molar equivalents). The reason may be as follows. The solubility of sucrose in pyridine is lower than in DMF, and excess pyridine-SO_3 will be present on the surface of undissolved sucrose. The resulting pyridinium salt is also insoluble in pyridine, and may cover the new surface of unreacted sucrose, resulting in persulfation.

Treatment of the barium salt of sucrose octasulfate with Amberlite IR-120 (K^+, Rb^+, Cs^+, or NH_4^+) resin gave the corresponding crystalline salt. The crystalline potassium salt of sucrose octasulfate was obtained in two forms, needles (K–I) and prisms (K–II), by different recrystallization procedures. Table II shows the elemental analysis data for both forms and for other crystalline salts. The results are in accord with the theoretical values, and the presence of water of crystallization was confirmed by thermogravimetry.

Approximate determination of the molecular weights of the ammonium and potassium (K–I and K–II) salts by an X-ray diffraction procedure,[8] and assignment of the ^{13}C-nuclear magnetic resonance signals of the octasulfate supported the sucrose octasulfate salt structure.

Treatment of the basic aluminium salt of sucrose sulfate prepared in the preceding work[2,3] with potassium hydroxide gave a potassium salt in almost quantitative yield; this was identical with the crystalline potassium salt of sucrose octasulfate. Thus, the sugar moiety of the basic aluminium salt should be sucrose octasulfate.

Experimental[11]

Sulfation in Dimethylforamide——Finely powdered sucrose (10 g) was dissolved in dry DMF (200 ml) by warming at 60°, and a calculated amount of pyridine-SO_3, prepared from pyridine and SO_3, was added to the solution. The mixture was stirred for 10 hr at 60°. After cooling, the reaction mixture was poured into Et_2O (1000 ml), and the resulting precipitate of the pyridinium salt was dissolved in H_2O (100 ml). An aqueous solution of $Ba(OH)_2$ was added dropwise to this solution to adjust it to pH 8, and the precipitate of $BaSO_4$ was removed by filtration. Concentration of the filtrate gave the barium salt of sucrose sulfate as an amorphous powder from EtOH. Yield: g (molar ratio of pyridine-SO_3 to sucrose); 15.7 (3), 25.0 (5), 41.9 (9), 47.9 (11).

Sulfation in Pyridine——Sucrose (10 g, 0.029 mol) was added to a solution of pyridine-SO_3 (23.3 g, 0.146 mol) in pyridine (100 ml), and the mixture was stirred at 60° for 4 hr. After cooling, excess pyridine was evaporated off and the residue of pyridinium salt was dissolved in H_2O (100 ml). Treatment as described above gave the barium salt of sucrose octasulfate (21.8 g) as an amorphous powder from EtOH. The barium salt (23.7 g) was also obtained using 9 molar equivalents of pyridine-SO_3 under the same conditions.

Sodium Salt of Sucrose Octasulfate——A solution of the barium salt of sucrose octasulfate (2.0 g) in H_2O (50 ml) was passed through a column of Amberlite IR-120 (Na^+) (30 ml). The eluate was concentrated to afford an amorphous powder of the sodium salt of sucrose octasulfate (1.6 g).

Crystalline Salts of Sucrose Octasulfate——Treatment of the barium salt of sucrose octasulfate on a column of Amberlite IR-120 (K^+, Cs^+, Rb^+, or NH_4^+) as described above gave the corresponding salt of sucrose octasulfate as crystals from MeOH. Recrystallization of each salt from aqueous MeOH gave colorless needles. On recrystallization of the potassium salt, needles (K-I, from aqueous MeOH) and prisms (K-II, from H_2O) were obtained. Analytical values are given in Table II.

Sodium Salt of Sucrose 1′,6′,6-Trisulfate——3′,4′,2,3,4-Penta-O-acetylsucrose (3.5 g) was dissolved in pyridine (40 ml) and pyridine-SO_3 (6.6 g) was added to this solution. The mixture was stirred at room temperature overnight. H_2O (100 ml) was added, and an aqueous solution of $Ba(OH)_2$ was added dropwise to adjust the pH to 8. The precipitate of $BaSO_4$ was removed by filtration, the filtrate was concentrated,

8) The crystal structure of the potassium salt of sucrose octasulfate was determined by X-ray diffraction and the results will be reported shortly.

9) A part of this work was presented at the 96th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April, 1976. The details of the assignment will be fully reported later.

11) All concentrations were carried out by evaporation under reduced pressure, keeping the bath temperature below 40°.

and the residue was dissolved in MeOH (50 ml). Treatment of the solution with ammonia for 1 hr under ice cooling, followed by concentration gave the barium salt of sucrose 1′,6′,6-trisulfate (1.8 g) as an amorphous powder from EtOH. Treatment of the barium salt on a column of Amberlite IR-120 (Na^+) as described above gave the sodium salt of sucrose 1′,6′,6-trisulfate (1.38 g) as an amorphous powder from EtOH; the product contained a small amount of disulfate, detectable by paper electrophoretic analysis.[6] The analytical values are given in Table I.

[Chem. Pharm. Bull. 28(2) 641—644 (1980)]

A Novel Method for the Fluorometric Assay of Proteins using Hypochlorite-Thiamine Reagent[1]

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(Received May 18, 1979)

A fluorometric assay method for proteins based on the reaction of peptide groups is described. The N-H groups of peptides are chlorinated with sodium hypochlorite, and the resulting N-chloropeptides are allowed to react with thiamine, giving fluorescent thiochrome. The present method is highly sensitive and is applicable to 1—10 μg of protein. Little variability of fluorescence intensity was observed among proteins.

Keywords——protein, fluorometric determination; peptide, N-chlorination; fluorometric determination, protein; thiamine; thiochrome; sodium hypochlorite; determination of protein, fluorescence

Recent progress in fluorometry has made possible the ultramicroanalysis of proteins by the fluorescamine method,[3] the O-phthaldialdehyde method[4] and the dansyl chloride method.[5] This method are sensitive and simple, but they are based on the reaction of amino groups and are therefore affected by the content of amino groups in proteins.

In 1962, Mazur *et al.*[6] proposed a method for the detection of peptides through N-chlorination, followed by the oxidation of benzidine by the N-chloropeptides to give a blue color. This method was modified by Sandford *et al.*[7] for the determination of amides and peptides using amylose-iodide reagent, which is less hazardous than benzidine.[8] Since this method is based on the reaction of N–H groups, the fluorescence intensity reflects the amount of peptide groups. Thus, Sandford's method is less influenced by various amino acid residues,

1) This paper constitutes part VIII of a series entitled "Microanalysis of Proteins and Peptides." Preceding paper, Part V: Ref. 10. A preliminary account of this work has been presented: T. Kinoshita, J. Murayama, and A. Tsuji, *Chem. Pharm. Bull.*, **24**, 2901 (1976).

2) Location: *a) 5-9-1, Shirokane, Minato-ku, Tokyo 108, Japan; b) 1-5-8, Hatanodai, Shinagawa-ku, Tokyo 142, Japan; c) 3-37-1, Sakashita, Itabashi-ku, Tokyo 174, Japan.*

3) *a)* S. Udenfriend, S. Stein, P. Böhlen, W. Leimgruber, W. Dairman, and M. Weigele, *Science*, **178**, 871 (1972); *b)* S. Stein, P. Böhlen, W. Dairman, and S. Udenfriend, *Arch. Biochem. Biophys.*, **155**, 202 (1973); *c) Idem, ibid.*, **155**, 213 (1973).

4) M. Roth, *Anal. Chem.*, **42**, 880 (1971).

5) T. Kinoshita, F. Iinuma, and A. Tsuji, *Chem. Pharm. Bull.*, **22**, 2413 (1974).

6) R.H. Mazur, B. Ellis, and P.S. Cammerata, *J. Biol. Chem.*, **237**, 1619 (1962).

7) P.A. Sandford, A.J. Nafziger, and A. Jeanes, *Anal. Biochem.*, **42**, 422 (1971).

8) D.P. Griswald and J. Weisburger, *Cancer Res.*, **28**, 924 (1968).