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INTRODUCTION OF AMINO GROUPS INTO CELLULOSE VIA (2, 3-DIBROMOPROPYL)CELLULOSE UNDER MILD CONDITIONS

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ABSTRACT: A technique to derivatize cellulose under mild conditions was devised. First, double bonds (allyl groups) were introduced into cellulose, and then the allylcellulose was converted to (2,3-dibromopropyl)cellulose. The dibromo derivative was fairly stable and could be stored for more than 6 months at -20°C . Amino compounds reacted on (2,3-dibromopropyl)cellulose at 37°C , and covalent bonds were formed between them. The cellulose derivatives prepared by this technique include [3-amino(or alkylamino)-2-bromopropyl]cellulose, [3-(ω -aminoalkylamino)-2-bromopropyl]cellulose, [3-(2-phosphoxyethylamino)-2-bromopropyl]cellulose, [3-anilino(or sulfanilino)-2-bromopropyl]cellulose. Cytochrome c_3 and flavin-adenine dinucleotide were combined to cellulose by this technique. The degree of substitution of the above derivatives depended on the type of amine, and was 0.38 when hexylamine was the ligand. Ligands having a thiol group was bound to cellulose at 100°C . Some of these compounds had affinity for copper ions and ionic dyes, ion-exchange capacity, and resistance to cellulase. The cytochrome c_3 -combined cellulose had biochemical reactivity to transfer electrons to produce hydrogen gas from protons in a medium by the catalytic action of hydrogenase. The activity of 10 mg of the cytochrome c_3 -combined cellulose corresponded to 0.33 nmol of free cytochrome c_3 .

1. INTRODUCTION

A number of techniques to derivatize cellulose for various applications have been devised. Conventional derivatizations endowed cellulose with some improved characteristics such as dyeability, thermoplasticity, solubility, etc. Cellulose can also be endowed with more sophisticated functions such as ion exchange-ability, catalytic activity, affinity for specific biological materials, permeability for specified substances, etc. The cyanogen bromide activation method introduced by Axen *et al.* [1] is used in industrial field, but the ligation of the ligand molecule by this technique involves the formation of isoureido bonds, which are cleaved slowly, especially in the presence of nucleophiles such as amine [2-4]. Sato *et al.* [5] prepared *N*-(3-isocyanato-4-methylphenyl)carbamoyl]cellulose, to which amino acid esters could be introduced in high yields, but the reaction was carried out in organic solvent. Chlorodeoxycellulose is another starting material to prepare functionalized derivatives [6,7]. Nakamura *et al.* [7] succeeded in chemical in-

troduction of organic amines to cellulose using 6-chloro-6-deoxycellulose, but the reaction was carried out at temperature higher than 100°C . Yoshimura [8] introduced allyl groups to the cellulose, and prepared crosslinked derivatives with vinyl monomers [9]. Isogai *et al.* [10] and Kondo *et al.* [11,12] succeeded in complete allylation of cellulose. Plisko *et al.* [13] brominated the unsaturated groups of allyl- and methallylcelluloses, but they did not attempt to prepare derivatives from the brominated celluloses.

In order to functionalize cellulose with biomolecules which lose activity at higher temperature or in organic solvents, the authors tested several functional groups and found that the dibromopropyl groups attached to the cellulose could be substituted with various amines in aqueous media. This paper introduces a new technique to prepare cellulose derivatives, having a stable $-\text{CH}_2\text{-NH}-$ linkages, under mild conditions.

2. EXPERIMENTAL

2.1 Materials

Cellulose powder (200-300 mesh pulverized filter paper, Toyo Roshi, Tokyo) was boiled in 0.5% NaOH for 20 min, washed with distilled water several times, and then dried.

Hydrogenase [Enzyme Code: 1.12.2.1][14] and cytochrome c_3 [15] were purified from *Desulfohalobium vulgare* Miyazaki (a species of sulfate-reducing bacteria), according to the reported procedures.

Amines were commercial products. Aqueous spermine solution (12% as free base), was prepared by neutralizing spermine tetrahydrochloride with aqueous NaOH. Aqueous *O*-phosphorylethanolamine (20%, pH 11.2), arginine (5%, pH 11.7), sodium aspartate (4.5%, pH 11.0), and sodium sulfanilate (16%, pH 11.0) were used. pH of these solutions had been adjusted with aqueous NaOH. Cytochrome c_3 (0.08 mM dissolved in 10 mM Na_3PO_4 containing 0.05% NaN_3) and flavin-adenine dinucleotide (FAD) (84 mM in 50 mM Na_3PO_4 containing 0.05% NaN_3) were used as biomolecules.

2.2 Analysis

2.2.1 Infrared Spectra

Infrared spectra were collected using a FTIR spectrometer (Perkin-Elmer Co., Type 1720X). A 2-mg portion of a sample blended with 150 mg of spectral grade KBr powder was compressed into a disk, and subjected to the FTIR spectroscopy. For each sample, 20 times scanings and a 2 cm^{-1} step were used.

2.2.2 Degree of Substitution (DS)

DS of some cellulose derivatives was determined by elementary analysis of the cellulose sample dried *in vacuo*. Since the cellulose is hygroscopic and absorbs humidity during weighing, the best fit estimates for DS and water content were calculated and given in the text. Phosphorus was analyzed by means of an inductivity coupled argon plasma atomic emission spectrophotometer (Jarrell ash, ICAP-575), after the cellulose was digested by boiling with sulfuric acid containing a drop of nitric acid. DS of aliphatic aminated cellulose derivatives was determined using a pH meter by titrating the suspension of a sample with 0.01 M H^+ (5 mM H_2SO_4). The inflection point of the titration curve (pH curve) was assumed to be the end point.

2.2.3 Concentrations of Metal Ions and Dyes

Metal ions (Cu^{2+} , Co^{2+} , Cd^{2+} , Ni^{2+} , Zn^{2+}) were

determined with an atomic absorption spectrophotometer (Jarrell ash, Type AA-I MK-II). The amount of dye adsorbed on cellulose derivative was determined from the dye concentration in the solution before and after the dyeing treatment. Dye concentration was determined from the maximum absorption (A_{max}) in UV-visible spectra (Shimadzu, UV-240).

2.2.4 Biochemical Reactivity

Biochemical reactivity of cytochrome c_3 -combined cellulose as an electron carrier for hydrogenase was measured using the hydrogen evolution technique [16]. A reaction mixture containing 0.3 nmol hydrogenase and either the cytochrome c_3 -combined cellulose or natural cytochrome c_3 in 2.9 mL of 20 mM phosphate buffer, pH 7.0, was placed in the main compartment of a vessel (vol: 15 mL) of Warburg manometer. The side arm contained 0.1 mL of sodium dithionite solution (4% in 0.2 M disodium hydrogen phosphate). The gas phase was pure nitrogen. The content in the side arm was dipped into the main compartment, and from this point the rate of hydrogen evolution was assayed in terms of increase in the gas pressure.

2.2.5 The Stability of Cellulose Derivatives to the Cellulase Digestion

Crude cellulase prepared from *Trichoderma viride* [17] was dissolved (0.94 mg/mL) in 20 mM acetate buffer, pH 5.0, centrifuged for 20 min at 5000 rpm to remove turbidity, and used as the cellulase solution. The absorbance of this solution at 280 nm was 0.971. Cellulose derivative (8.0 mg) was suspended in 8.0 mL of the cellulase solution, and the suspension was treated at 37 °C with vigorous stirring for 3 h. At the end of the treatment, the mixture was allowed to stand to precipitate the cellulose derivative, and the amount of the reducing sugar in the supernatant was determined by the method of Somogyi [18] and Nelson [19].

2.3 Derivatization of Cellulose

2.3.1 Preparation of Allylcellulose

Cellulose powder (30 g) was suspended in 450 mL of 18% NaOH for 1.5-2 h at 22 °C for mercerization, and filtered by suction to remove an excess of the alkali solution. The mercerized cellulose was suspended in 320 g of 2-propanol containing 8 mL of aqueous 10% NaOH and allowed to stand for 2 h at 4

°C. After allyl chloride (80 g) was added to the mixture, it was refluxed with stirring. Effective mixing was required to prepare uniformly substituted cellulose derivative by the reaction of allyl halide with mercerized cellulose [8]. To observe the refluxing time dependence of allylation, aliquots of the cellulose powder were taken out at intervals, filtered with a glass filter, washed repeatedly with cold H₂O to remove alkali and the solvent, and stored in a cold room under nitrogen. At the end of refluxing for 20 h, the mixture was filtered, washed with cold H₂O, and stored in a cold room under nitrogen. The product after 20 h-reflux, for example, was named allylcellulose-20. The yield of allylcellulose-20 was 30%. The IR spectrum of allylcellulose-20 had sharp absorption band around 1640 cm⁻¹ due to stretching of C = C double bonds (Fig. 1b). Mercerized cellulose had a broad absorption around this wavenumber in the IR spectrum, and this is ascribable to adsorbed water (Fig. 1a). Since allylcellulose is oxidized under air at room temperature [11,20], allylcellulose samples were stored either at 4 °C under nitrogen or at -20 °C, or brominated promptly.

2.3.2 Bromination of Allylcellulose

To aqueous suspensions of various allylcellulose samples, an alcoholic bromine solution (8%) was added until the color of bromine persisted in the mixture [13]. The brominated cellulose was washed thoroughly with distilled water, and dried *in vacuo*. The bromo derivative, i.e., *O*-(2,3-dibromopropyl)cellulose prepared from allylcellulose-20, for example, was named (dibromopropyl)cellulose-20, in this paper. The bromination of allylcellulose proceeded quantitatively, and the yield was nearly 100%. Elementary analysis (Table 1) suggested that (dibromopropyl)cellulose-9 and (dibromopropyl)cellulose-20 had the structure of *O*-(2,3-dibromopropyl)cellulose with DS of 0.15 and 0.37, respectively.

The bromo derivatives of cellulose were also prepared by the reaction of allylcellulose with aqueous bromine solution (8% in 2% NaOH solution). The brominated cellulose thus prepared had the same elementary composition to that of the (2,3-dibromopropyl)cellulose prepared by reaction of allylcellulose with alcoholic bromine solution (Table 1). Both of the brominated cellulose derivatives gave

Fig. 1. FTIR spectra of some cellulose derivatives. (a) mercerized cellulose, (b) allylcellulose, (c) hxdm-cellulose, (d) arginine-combined cellulose, (e) sulfanilic acid-combined cellulose, and (f) thioglycolic acid-combined cellulose. A 2-mg portion of a sample blended with 150 mg of spectral grade KBr powder was compressed into a disk, and subjected to the FTIR spectroscopy.

identical products upon the reaction with amines.

2.3.3 Reaction of Amines with

O-(2,3-Dibromopropyl)cellulose

The (dibromopropyl)cellulose was suspended in aqueous amines or liquid amines, and allowed to stand at 37 °C for 20 h. The mixture was filtered, washed thoroughly with water, and dried *in vacuo*. In the case of the reaction with cytochrome c₃, the product was washed thoroughly with H₂O, with 0.1 M ammonia, with H₂O again, and dried *in vacuo*.

3. RESULTS

3.1 Reaction Products of (2,3-Dibromopropyl)cellulose With Amines

3.1.1 Reaction Product with Hexamethylenediamine (hxdm-cellulose)

Elementary analysis of the hexamethylenediamine

Table 1 Elementary Analysis of Cellulose Derivatives

		C (%)	H (%)	N (%)	Br (%)
(dibromopropyl)cellulose-9	found	38.30	5.85		11.61
	calcd (1)	38.31	5.84		11.85
(dibromopropyl)cellulose-20 ^a	found	35.18	5.17		24.79
(dibromopropyl)cellulose-20 ^b	found	35.16	5.20		23.54
	calcd (2)	35.09	5.09		24.29
hxdm-cellulose-9	found	43.52	7.26	2.34	—
	calcd (3)	42.96	6.78	2.26	
hxdm-cellulose-20	found	43.99	7.08	3.10	8.29
	calcd (4)	43.65	6.93	3.05	8.71
aniline-combined cellulose	found	36.52	5.34	0.39	20.75
	calcd (5)	36.64	5.31	0.40	21.69
sulfanilic-acid combined cellulose	found	36.67	5.67	0.79	
	calcd (6)	36.57	5.30	0.80	

^a Prepared by the reaction of allylcellulose-20 with alcoholic bromine solution

^b Prepared by the reaction of allylcellulose-20 with aqueous bromine solution

Basis of the calculation

- (1) for *O*-(2,3-dibromopropyl) group with DS of 0.15 and 5% H₂O
- (2) for *O*-(2,3-dibromopropyl) group with DS of 0.37 and 3% H₂O
- (3) for 3-(6-aminohexylamino)-2-bromopropyl group with DS of 0.17 and 4% H₂O
- (4) for 3-(6-aminohexylamino)-2-bromopropyl group with DS of 0.26, dihydroxypropyl group with DS of 0.11, and 3% H₂O
- (5) for 3-anilino-2-bromopropyl group with DS of 0.07, dibromopropyl group with DS of 0.30, and 4% H₂O
- (6) for 3-sulfanilino-2-bromopropyl group with DS of 0.15, dibromopropyl group with DS of 0.22, and 5% H₂O

derivative (hxdm-cellulose) prepared from (dibromopropyl)cellulose-20 suggested that the product had the structure of [3-(6-aminohexylamino)-2-bromopropyl] cellulose with some debrominated side chains (Table 1). The amount of hexamethylenediamine combined to cellulose determined either by elementary analysis or by titration corresponds to 0.26 DS of aminohexylamino groups. The amine was not removed from the cellulose by boiling the derivative in water for 20 min.

DS of hxdm-cellulose as a function of the reaction time of allylation is shown in Fig. 2. Treatment of the (dibromopropyl)cellulose-20 with hexamethylenediamine in aqueous solution of different concentrations or in alcoholic solution gave aminohexylamino derivatives of different DS as shown in Table 2.

The FTIR spectrum of hxdm-cellulose (Fig. 1c) had absorption bands around 1640 and 1570 cm⁻¹ due

to N-H bending, and a characteristic absorption band around 1460 cm⁻¹ due to CH₂ bending mode of (CH₂)_n.

The kinetics of aminohexylation of (dibromopropyl)cellulose-20 with 20% hexamethylenediamine at 37°C is shown in Fig. 3. The reaction was nearly completed in 60 min.

3.1.2 Reaction Product with Ammonia (amino-cellulose)

DS of amino-cellulose-20 prepared from (dibromopropyl)cellulose-20 was 0.26 on the assumption that the amination proceeded to produce (3-amino-2-bromopropyl)cellulose. Changes in DS of amino-cellulose as a function of the reaction time of the allylation are shown in Fig. 2.

3.1.3 Reaction Product with Hexylamine (hexylamine-cellulose)

DS of hexylamine-cellulose-20 prepared from (di-

Fig. 2. Degree of substitution of the cellulose derivatives in terms of refluxing time of allylation. During the allylation of cellulose, aliquots of the cellulose powder were taken out at intervals, filtered, washed, and brominated to (2,3-dibromopropyl) celluloses of different DSs, which were determined by elementary analysis. Some of the (2,3-dibromopropyl) celluloses were aminated and DSs of the products were determined either by elementary analysis or titration. (○), hxdm-cellulose; (●), (dibromopropyl) cellulose; (△), amino-cellulose; (▲), hexylamine-cellulose.

bromopropyl)cellulose-20 was 0.38 on the assumption that the amination proceeded to produce (3-hexylamino-2-bromopropyl) cellulose. Changes in DS of hexylamine-cellulose as a function of the reaction time in the allylation step are shown in Fig. 2.

3.1.4 Reaction Products of (Dibromopropyl) cellulose-20 with Other Amines

The FTIR spectra of the aminated celluloses had absorption bands around 1640 and 1570 cm^{-1} due to N-H bending. The FTIR spectrum of the arginine-combined cellulose (Fig. 1d) has high intensity absorption bands around 1675, 1618, and 1577 cm^{-1} , which corresponded to those of L-arginine. The FTIR spectrum of the sulfanilic acid-combined cellulose (Fig. 1e) suggests the presence of benzene ring in the product. The results of elementary analysis of aniline- and sulfanilic acid-combined celluloses are given in Table 1. The cytochrome c_3 -combined cellulose had the color of cytochrome c_3 (light red), and the FAD-combined cellulose had the color of the flavin compounds (yellow).

Ethylenediamine: Cationic groups of 3.8 meq/g were introduced into cellulose. This value corres-

Fig. 3. The time course curve of the reaction of hexamethylenediamine with (dibromopropyl)cellulose to produce [3-(6-aminoethylamino)-2-bromopropyl] cellulose (hxdm-cellulose). (Dibromopropyl)cellulose-20 was mixed with 20% hexamethylenediamine solution and incubated at 37 °C. At intervals, aliquots were taken out from the mixture, washed immediately with water, and dried. The content of the amino groups was estimated by titration with 5 mM H_2SO_4 , and the degree of substitution per anhydroglucose unit was calculated.

ponds to 0.43 2-aminoethylamino groups per anhydroglucose unit, on the assumption that the reaction product has 3-(2-aminoethylamino)-2-bromopropyl and 2,3-bis(2-aminoethylamino)-propyl groups. Treatment with aqueous 20% ethylenediamine resulted in the introduction of 2.0 meq/g cationic groups, or DS 0.22 of 2-aminoethylamino group to the cellulose.

Polyethylenimine: Cationic groups of 2.2 meq/g were introduced into cellulose. Since this amine has several kinds of groups to react with cellulose, DS could not be estimated. DS and cationic density of the cellulose derivatives treated with various amines are summarized in Table 2.

3.2 Properties of Aminated Celluloses

3.2.1 Affinity for Metal Ions

The polyethylenimine-combined cellulose (10 mg each) was added to 10 mL each of copper sulfate solutions with different concentrations (0.01, 0.02, 0.05, or 0.1 mM), allowed to stand overnight, and then filtered with a glass filter. The copper was not detectable in the filtrate in every case.

Various cellulose derivatives (10 mg each) were added to 10 mL of 0.2 mM copper sulfate, cadmium nitrate, cobalt chloride, nickel sulfate, zinc acetate, or mixed solution containing 0.2 mM each of these metal

Table 2 Degree of Substitution of Amino Groups Introduced into Cellulose^a

Amine used	State of amine	Degree of substitution	Cationic density meq/g	Method of analysis ^b
Ammonia ^c	aq. 28%	0.26	1.26	T
Hexylamine	aq. 50%	0.38	1.56	T
Ethylenediamine	100%	0.43	3.8 ^c	T
Ethylenediamine ^c	aq. 20%	0.22	2.0 ^c	T
Hexamethylenediamine	aq. 60%	0.38	3.04	T
Hexamethylenediamine ^c	aq. 20%	0.26	2.27	T and E
Hexamethylenediamine	aq. 5%	0.20	1.67	T
Hexamethylenediamine	alc. 20%	0.23	2.04	T
Spermine	aq. 12%	0.13	2.1 ^c	T
Polyethylenimine ^c	aq. 10%		2.2 ^c	T
Ethanolamine	aq. 20%	0.31	1.30	T
Phosphoethanolamine	aq. 20%	0.16		P
Tris base ^{dc}	aq. 20%	0.20	0.86	T
Piperidine ^c	aq. 20%	0.42	1.80	T
Morpholine	aq. 20%	0.18	0.8 ^c	T
Aniline	100%	0.07		E
Sulfanilate	aq. 16%	0.15		E

^a (Dibromopropyl)cellulose-20 (2,3-dibromopropyl groups, DS = 0.37) was immersed in amine for 20 h at 37 °C.

^b Abbreviations are: E, elementary analysis; P, phosphorus content; and T, acid-base titration.

^c Products were used in experiments shown in Tables 3, 4, or 5.

^d Tris base is 2-amino-2-hydroxymethyl-1, 3-propanediol.

^e The pH curve for the titration was not sharp, and hence the endpoint of the titration may not be accurate.

Table 3 Adsorption of Metal Ions to Cellulose Derivatives

Cellulose derivatives	Cu ²⁺		Cd ²⁺		Co ²⁺		Ni ²⁺		Zn ²⁺	
	Alone*	Mixt**	Alone*	Mixt**	Alone*	Mixt**	Alone*	Mixt**	Alone*	Mixt**
	(mmol/g)		(mmol/g)		(mmol/g)		(mmol/g)		(mmol/g)	
Mercurized	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00
Amino- ^a	0.17	0.20	0.00	0.02	0.00	0.00	0.02	0.02	0.00	0.01
Ethylenediamine- ^a	0.19	0.19	0.06	0.04	0.03	0.04	0.10	0.08	0.07	0.04
Hexamethylenediamine- ^a	0.20	0.20	0.00	0.02	0.01	0.02	0.03	0.07	0.01	0.07
Polyethylenimine- ^a	0.18	0.19	0.06	0.05	0.03	0.01	0.08	0.05	0.03	0.03

^a The cellulose derivatives listed in Table 2 were used.

Cellulose derivatives (10 mg each) were added to 10 mL each of 200 μ M solutions of metal ions (* only one kind of metal ion, ** mixture of Cu²⁺, Cd²⁺, Co²⁺, Ni²⁺, and Zn²⁺), and allowed to stand overnight with occasional shaking. After precipitation of the cellulose derivatives, the metal ion concentration of the supernatant was measured, and the amount of metal ions adsorbed on the cellulose derivative was calculated.

ions, and allowed to stand overnight with occasional shaking. The solution was allowed to stand to precipitate the cellulose derivatives, and the concentrations of the metal ions in the supernatant was measured. The results are shown in Table 3. The aminated derivatives had strong and specific affinity to copper ion, whereas mercerized cellulose had no affinity to any metal ions.

3.2.2 Dye Adsorption

The capacity of cellulose derivatives to adsorb dye was tested with two kinds of dyestuffs: methyl orange ($A_{\max} = 460$ nm), an acid dye, and Toluidine Blue O ($A_{\max} = 630$ nm), a basic dye. The concentration of dye in the dye bath was 40 mg/L, and the bath ratio was 500 : 1. No coagent was added to the bath. The cellulose sample was immersed in the dye bath, and allowed to stand at 37 °C for 20 h. The sample was removed from the bath by filtration with PTFE membrane filter unit (Millex-SR, porosity: 0.5 μ m), and the absorbance of the filtrate was measured. The derivative had adsorptive capacity much higher than that of mercerized cellulose, as shown in Table 4.

3.2.3 Biochemical Reactivity of Cytochrome c_3 -Combined Cellulose

The cytochrome c_3 -combined cellulose (26 mg) had biochemical reactivity corresponding to 0.86 nmol of cytochrome c_3 as an electron carrier for hydrogenase, i.e., 10 mg of the cytochrome c_3 -combined cellulose corresponds to 0.33 nmol free cytochrome c_3 . After the hydrogen evolution test was terminated, the reaction mixture was filtered. No cytochrome c_3 was detected in the filtrate.

3.2.4 Stability of Cellulose Derivatives to the Cellulase Digestion

As shown in Table 5, the cellulose derivatives having cationic or anionic groups were more resistant to the action of cellulase than those having nonionic groups.

3.3 Reaction of (2,3-Dibromopropyl)cellulose with Thiol

The (dibromopropyl)cellulose-20 was suspended in 2 M buffer solution of sodium mercaptoacetate (pH 9), and allowed to stand at 37 °C for 20 h. No carboxyl groups were introduced into cellulose. The mixture of the same composition was heated at 100 °C under reflux conditions for 4 h, washed with 0.1 M HCl and

Table 4 Dye Absorption to Some Cellulose Derivatives

Cellulose derivatives	Dyestuff	
	Methyl Orange	Toluidine Blue O
	(acid dye)	(basic dye)
	(mg/g)	(mg/g)
Mercerized	10.05	4.45
(Dibromopropyl)cellulose-20	—	7.16
Amino- ^a	19.60	—
Hxdm- ^a	19.78	—
Polyethylenimine- ^a	19.92	—

^a The cellulose derivatives listed in Table 2 were used.

Table 5 Susceptibility of Cellulose Derivatives to the Degradation by Cellulase

Cellulose derivatives	Susceptibility to cellulase relative to that of mercerized cellulose (%)
Mercerized	100
Allylcellulose-20	60
(Dibromopropyl)cellulose-20	25
Amino- ^a	6
Ethylenediamine- ^a	5
Hxdm- ^a	2
Polyethylenimine- ^a	2
Piperidine- ^a	14
Tris- ^a	9
Sulfanilic- ^b	10
Thioglycolic- ^b	2
DE-32 ^c	5

^a The cellulose derivatives listed in Table 2 were used.

^b Preparation of the sulfanilic and thioglycolic acids-combined cellulose derivatives are described in the text.

^c Diethylaminoethylcellulose (Whatman) having ionic density of 1 meq/g.

The cellulose derivative (8 mg) was suspended in 8 mL of the cellulase solution (0.94 mg/mL dissolved in 20 mM acetate buffer, pH 5.0), and the mixture was treated at 37 °C with vigorous stirring. After 3 h, the mixture was allowed to stand to precipitate the cellulose derivatives, and the concentration of the reducing sugar in the supernatant was determined. About 2.3 μ mol/mL of reducing sugar was released from the mercerized cellulose under these conditions.

H₂O, and dried. Introduction of carboxyl groups to the cellulose was confirmed by FTIR measurement as shown in Fig. 1(f), and a band at 1727 cm⁻¹ due to C=O stretching was clearly detected. The carboxyl content determined by titration with 50 mM NaOH was about 0.19 per anhydroglucose unit.

3.4 Storage of (2,3-Dibromopropyl)cellulose

Storage of (dibromopropyl)cellulose-20 for 6 months at room temperature resulted in slight coloration of the derivative. On the other hand, (dibromopropyl)cellulose-20 could be stored for 6 months at -20 °C without any change by visual inspection. The reaction of hexylamine and hexamethylenediamine with the (dibromopropyl)cellulose-20 stored at -20 °C gave products which had cationic densities identical with those produced from hexylamine and hexamethylenediamine with the freshly prepared (dibromopropyl)cellulose-20, respectively.

4. DISCUSSION

Allylcellulose has been spotlighted as a versatile starting material for preparing various cellulose derivatives [8-12], but is rather unstable and deteriorates under air at ambient temperature [11,20], and no biomolecules have been incorporated directly to allylcellulose. In this study new technique was devised to incorporate amino compounds including biomolecules into cellulose via (2,3-dibromopropyl)cellulose prepared by bromination [13] of allylcellulose. This technique has the following advantages; 1) The starting material, (2,3-dibromopropyl)cellulose, is fairly stable and could be stored without deterioration at -20 °C. 2) Various kinds of ligand molecules can be introduced. 3) Cationic density to be introduced to cellulose is controllable by choosing the ligand compound. For example, cationic cellulose derivative with an ionic density of 2.3 meq/g can be prepared by reacting the (dibromopropyl)cellulose (DS = 0.37) with a diamine (hexamethylenediamine), and that of 1.3 meq/g of cationic group can be prepared by reacting the same (dibromopropyl)cellulose with a monoamine (ethanolamine or ammonia).

The reaction of (dibromopropyl)cellulose (DS = 0.37) with various amines shows that some bromine atoms remained on the cellulose without being substituted with amine. The number of amines introduced

depended mainly on the basicity and the concentration, and not on the size, of the amines, e.g., comparison of the reaction products from piperidine ($pK_a = 11.2$), hexamethylenediamine ($pK_a = 10.9$), and ethylenediamine ($pK_a = 9.9$) at 20% (on the molar basis, the concentration of ethylenediamine is the highest of the three), those from hexamethylenediamine of different concentrations, or those from bulky Tris base ($pK_a = 8.3$) and less bulky morpholine ($pK_a = 8.5$) in Table 2. The reaction of the (dibromopropyl)cellulose with hexamethylenediamine proceeded very rapidly, and was completed within a few hours (Fig. 3).

Cellulose derivatives reacted with diamine or polyamine may be of special importance. They have strong and specific affinity for copper ion, and hence will be used for removal and recovery of copper ion. Slight differences in adsorption of copper ion probably reflected the cationic density of the derivatives. Other divalent metal ions were adsorbed, to any extent when the derivatives had the 'ethylenediamine' structure. The cellulose derivatives with amino groups have excellent capacity to be stained by dyestuff without coagents. These derivatives had extreme resistance to cellulase.

Cytochrome *c*₃ is a natural electron carrier for hydrogenase from *D. vulgaris* [21]: when it is reduced hydrogen is produced by the catalytic action of hydrogenase. The cytochrome *c*₃-combined cellulose still retained the biochemical reactivity, and this result indicates that the derivatization method introduced in this paper may be widely applicable to the field of biochemistry and biotechnology.

The (dibromopropyl)cellulose is fairly stable and may be used to react with most of biological materials having amino groups, as far as the materials are stable under weakly alkaline conditions. Some biomolecules are unstable even under weakly alkaline conditions and have to be reacted to the cellulose derivatives by the conventional cyanogen bromide technique [1]. For these unstable materials, hxdm cellulose may be useful as a supporting material, because this has long spacers with amino end groups which form more stable guanidino bonds [22], with ligand compound than isourea bonds produced by the ordinary cyanogen bromide technique.

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REFERENCES

1. R. Axen, J. Porath, and S. Ernback, *Nature* (London), **214**, 1302 (1967)
2. G. I. Tesser, H. U. Fisch, and R. Schwyzer, *Helv. Chim. Acta*, **57**, 1718 (1974)
3. T. Oka and Y. J. Topper, *Proc. Natl. Acad. Sci. USA*, **71**, 1630. (1974)
4. M. Wilchek, T. Oka, and Y. J. Topper, *Proc. Natl. Acad. Sci. USA*, **72**, 1055 (1975)
5. T. Sato, K. Karatsu, H. Kitamura, and Y. Ohno, *Kobunshi-Ronbunshu* **39**, 699 (1982)
6. T. Ishii, *Carbohydr. Res.* **154**, 63 (1986)
7. S. Nakamura, M. Amano, and Y. Saegusa, *Abstracts of Papers, 33rd IUPAC International Symposium on Macromolecules*, Montreal, PQ, Canada (1990), Session 1.8.6.
8. S. Yoshimura, *Sen'i Gakkaishi*, **21**, 317 (1965)
9. S. Yoshimura, *Sen'i Gakkaishi*, **21**, 358 (1965)
10. A. Isogai, A. Ishizu, and J. Nakano, *J. Appl. Polym. Sci.*, **29**, 3873
11. T. Kondo, A. Isogai, A. Ishizu, and J. Nakano, *J. Appl. Polym. Sci.*, **34**, 55 (1987)
12. T. Kondo, A. Ishizu, and J. Nakano, *J. Appl. Polym. Sci.* **35**, 885 (1988)
13. E. A. Plisko, V. A. Petrova, and G. A. Petropavlovskii, *Zh. Prikladnoi Khimii* **55**, 1101 (1982)
14. T. Yagi, K. Kimura, H. Daidoji, F. Sakai, S. Tamura, and H. Inokuchi, *J. Biochem.* **79**, 661 (1976)
15. J. G. Gayda, T. Yagi, H. Benosman, and P. Bertrand, *FEBS Lett.* **217**, 57 (1987)
16. N. Tamiya, Y. Kodo, T. Kameyama, and S. Akabori, *J. Biochem.* **42**, 613 (1955)
17. G. Okada, *J. Biochem.* **77**, 33 (1975)
18. M. Somogyi, *J. Biol. Chem.* **195**, 19 (1952)
19. N. Nelson, *J. Biol. Chem.* **153**, 375 (1944)
20. S. Yoshimura, *Sen'i Gakkaishi*, **21**, 410 (1965)
21. T. Yagi, M. Honya, and N. Tamiya, *Biochim. Biophys. Acta*, **153**, 699 (1968)
22. J. Schnapp and Y. Shalitin, *Biochem. Biophys. Res. Commun.*, **70**, 8 (1976)