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## Hydrophobic Chromatography of Tubulin on Immobilized Colchicine Columns with Various Spacers

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Eleven immobilized-colchicine adsorbents were synthesized from deacetylcolchicine (DAC) and Sepharose with various spacers (R) and their tubulin-adsorbing capacity was investigated. The adsorption capacity of DAC-R-Sepharose for tubulin depended on the hydrophobicity rather than the length of the spacer and was three to five times that of DAC-Sepharose without any spacer.

The results of the present studies can be summarized as follows. (1) The adsorbed tubulin showed two binding modes: a reversible binding which is abrogated at weak ionic strength (such as 0.1 M NaCl), and an irreversible binding which is abrogated by denaturants such as 6 M urea and 7 M guanidine-HCl. (2) The adsorbed tubulin was not eluted by colchicine but was eluted as colchicine-binding tubulin by 0.1 M NaCl. (3) Tubulin was adsorbed on hydrophobic ligands such as the phenyl, diphenyl, trimethoxyphenyl or naphthyl group. (4) Tubulin-colchicine complex was adsorbed by immobilized colchicine.

It is considered that the immobilized colchicine binds tubulin at a hydrophobic region, not necessarily at a specific colchicine-binding site.

**Keywords**—immobilized colchicine; tubulin; hydrophobic ligands; hydrophobic chromatography; various spacers

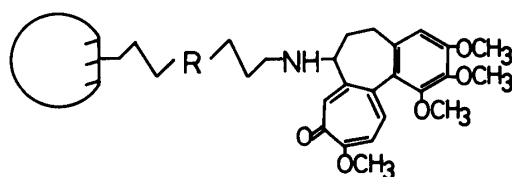
The reconstitution of microtubules *in vitro* was accomplished with a tubulin preparation obtained by the assembly-disassembly method of Shelanski *et al.*<sup>1)</sup> This assembly of tubulin, that is the formation of microtubules, is inhibited by specific binding of colchicine and this selective binding has been used for the isolation of tubulin from brain extract by affinity chromatography by some workers. Hinman *et al.*,<sup>2)</sup> Morgan and Seeds<sup>3)</sup> and Sandoval and Cuatrecasas<sup>4)</sup> tried to purify tubulin, by using a column of deacetylcolchicine (DAC) immobilized directly on BrCN-activated Sepharose, but the yield was low and this method still remains to be developed.

Cuatrecasas proposed that successful application of affinity chromatography in many cases would depend critically on placing the ligand at a considerable distance from the matrix backbone.<sup>5)</sup> In general, the separation efficiency is increased by the use of a spacer having a hexamethylene chain, which is hydrophobic in nature. The binding reaction between tubulin and colchicine in solution is slow and depends on temperature.<sup>6)</sup> Further, the binding reaction is believed to be hydrophobic in nature from thermodynamic data.<sup>7)</sup>

This paper describes the effects of spacers of various lengths and structures on the binding between tubulin and immobilized colchicine and further demonstrates the contribution of hydrophobic interaction of immobilized colchicine and the spacer with tubulin.

### Materials and Methods

**Materials**—The following chemicals were obtained from Tokyo Kasei Co., Ltd.:  $\gamma$ -amino-*n*-butyric acid,  $\epsilon$ -



Sepharose-spacer(R)-DAC

- group A [R:  $\omega$ -amino- $n$ -carboxyalkane]
- A-1  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{CO-DAC}$
- A-2  $\text{---NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO-DAC}$
- group B [R: succinyl- $\alpha,\omega$ -diaminodialkylamine]
- B-1  $\text{---NHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$
- B-2  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$
- group C [R: succinyl- $\alpha,\omega$ -diaminoalkane]
- C-1  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$
- C-2  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$
- C-3  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$
- C-4  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$
- C-5  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$
- C-6  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$
- C-7  $\text{---NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOCH}_2\text{CH}_2\text{CO-DAC}$

Fig. 1. List of DAC-R-Sepharoses with Various Spacers

The model structure of immobilized colchicine (DAC ligand, R spacer, Sepharose matrix) is indicated on top of the figure. DAC-R-Sepharoses are classified into three group based on the structure of the spacer, (A)  $\omega$ -amino- $n$ -carboxyalkane, (B) succinyl- $\alpha,\omega$ -diaminodialkylamine, (C) succinyl- $\alpha,\omega$ -diaminoalkane.

amino- $n$ -caproic acid, 2,2'-diaminodiethylamine, 3,3'-diaminodipropylamine, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, 1,7-diaminoheptane, 1,8-diaminooctane, 1,9-diaminononane and succinic anhydride. Bovine serum albumin, ovalbumin, chymotrypsinogen A and piperazine- $N,N'$ -bis(2-ethanesulfonic acid) (Pipes) were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden. Coupling reagent 3-(3-dimethylaminopropyl)1-ethylcarbodiimide $\cdot$ 2HCl was from Fluka, Buchs, Switzerland. Ampholine was obtained from LKB, Stockholm, Sweden. Colchicine and other reagents were bought from Wako Pure Chemicals, Tokyo.

**Preparation of DAC**—Deacetylation of commercial colchicine was performed by the method of Wilson and Friedkin.<sup>8)</sup> The reaction product was examined by thin-layer chromatography (TLC) and by ultraviolet (UV) spectrophotometry. It was found that the product was approximately a one to one mixture of deacetylcolchicine and isodeacetylcolchicine.

The structures of various DAC-R-Sepharoses are shown in Fig. 1. Based on the structure of the spacer (R), compounds (DAC-R-Sepharose) were classified into three groups, *i.e.*,  $\omega$ -amino- $n$ -carboxyalkane group (group A),  $\alpha,\omega$ -diaminodialkylamine group (group B) and  $\alpha,\omega$ -diaminoalkane group (group C), namely, two derivatives of  $\gamma$ -amino- $n$ -butyric acid (A-1) and  $\epsilon$ -amino- $n$ -caproic acid (A-2) as group A, two derivatives of 2,2'-diaminodiethylamine (B-1) and 3,3'-diaminodipropylamine (B-2) as group B, and seven derivatives of 1,3-diaminopropane (C-1), 1,4-diaminobutane (C-2), 1,5-diaminopentane (C-3), 1,6-diaminohexane (C-4), 1,7-diaminoheptane (C-5), 1,8-diaminooctane (C-6) and 1,9-diaminononane (C-7) as group C.

**Synthesis of DAC-R-Sepharose with Various Spacers**—Step 1. Coupling of Spacer Compounds to BrCN-Activated Sepharose: Cyanogen bromide-activated Sepharose 4B was prepared according to the method of Horigome *et al.*<sup>9)</sup> Each group A compound was dissolved in 10 ml 1% NaHCO<sub>3</sub>-NaOH solution at pH 10. Each of these solutions was added to 10 g of wet BrCN-activated Sepharose. On the other hand, 10 ml aliquots of 2 M group B and C compounds dissolved in an aqueous solution at pH 10 with concentrated HCl, were each added to 10 g of wet BrCN-activated Sepharose. The gel suspensions were adjusted to pH 10 with sodium hydroxide solution and mixed by end-over-end rotation for 16 h at 4°C. The unreacted spacer compounds were successively washed four times with 50 ml of deionized water, 1.0 M NaCl and finally deionized water.

Step 2. Succinylation of R-Sepharose: The nine kinds of aminoalkyl-Sepharoses of groups B and C were succinylated with succinic anhydride according to the method of Cuatrecasas.<sup>5)</sup> Solid succinic anhydride (10 mmol) was added to a mixture of 10 g of wet R-Sepharose and 10 ml of deionized water at pH 6.0, and the pH was maintained at 6.0 with 20% NaOH at 4°C. When no further change in pH occurred, the gel suspension was mixed by end-over-end rotation for 7 h at 4°C. The gel was washed with 0.1 M NaOH for 30 min and then with deionized water.

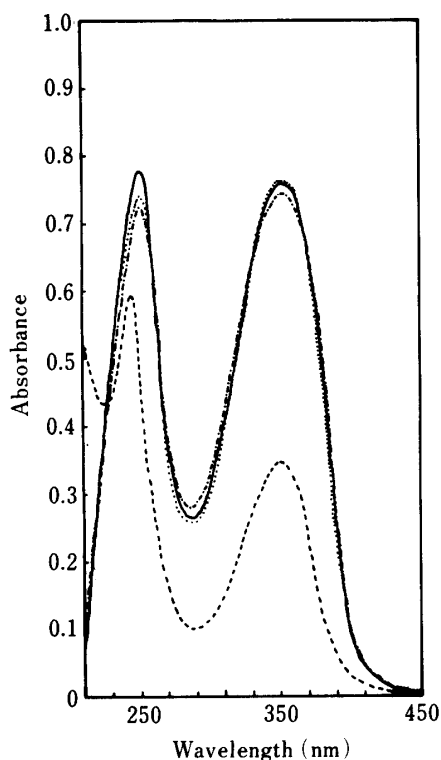


Fig. 2. Absorption Spectra of DAC-R-Sepharose

Curve A (—): DAC-succinyl-1,5-diaminopentane-Sepharose (80 mg wet weight) was suspended in 3 ml of 0.05% agarose solution and the absorption spectrum was measured against a suspension of R-Sepharose without DAC (80 mg wet weight) in 3 ml of 0.05% agarose solution. Curve B (---): DAC-succinyl-1,5-diaminopentane-Sepharose was packed in a column and washed with 30 ml of 6 M urea. The washed material was taken out and the absorption spectrum (80 mg wet weight in 3 ml of 0.05% agarose solution) was determined as described above. Curve C (· · · · ·): DAC-succinyl-1,5-diaminopentane-Sepharose was packed and washed with 30 ml of 7 M guanidine-HCl. The washed material was taken out and the absorption spectrum (80 mg wet weight in 3 ml of 0.05% agarose solution) determined. Curve D (- · - · -): Free colchicine (20  $\mu$ M) was dissolved in 95% ethanol and the absorption spectrum was measured.

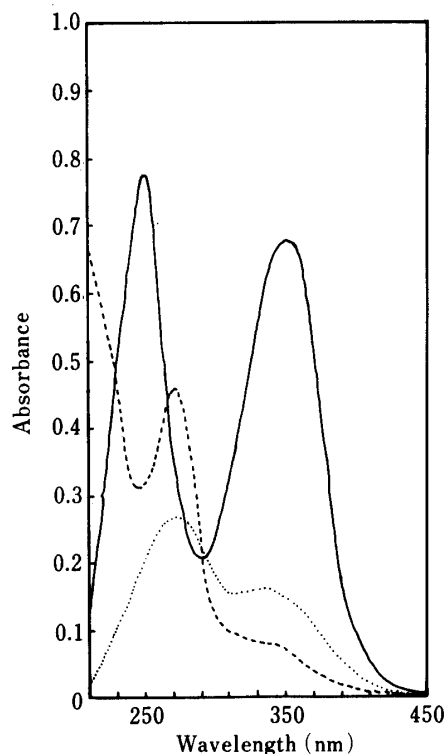


Fig. 3. Absorption Spectra of Lumicolchicine-R-Sepharose

Curve A (---): Lumicolchicine-succinyl-1,5-diaminopentane-Sepharose (70 mg wet weight) and curve B (—): DAC-succinyl-1,5-diaminopentane-Sepharose (70 mg wet weight) was suspended in 3 ml of 0.05% agarose solution, and the absorption spectra were determined as described in Fig. 2. Curve C (· · · · ·): Free lumicolchicine (20  $\mu$ M) was dissolved in 95% ethanol and the absorption spectrum was measured.

**Step 3. Coupling of DAC to R-Sepharose:** Coupling of DAC to eleven kinds of R-Sepharoses of groups A, B and C was performed in the presence of water-soluble carbodiimide as follows. A mixture of 10 g of wet R-Sepharose and 100 mg of DAC in 10 ml of 20% dioxane deionized water solution was adjusted to pH 4.7 and 200 mg of solid 3-(3-dimethylaminopropyl)1-ethylcarbodiimide·2HCl was added. The pH was maintained at 4.7, and the gel suspension was mixed by end-over-end rotation for 16 h at 4°C. The unreacted DAC was successively washed four times with 50 ml of 20% dioxane deionized water solution, 1 M NaCl and finally deionized water. The DAC-R-Sepharoses were stored in 0.05%  $\text{NaN}_3$ -1%  $\text{NaHCO}_3$  solution at 4°C. On the other hand, the synthesis of DAC-Sepharose without any spacer was carried out by the same procedure as described above, except for the coupling at pH 10.

The stability of DAC-R-Sepharose was examined by successive washings with 30 ml portions of 6 M urea and of 7 M guanidine-HCl. The absorption spectra and the ligand contents of DAC-R-Sepharose did not change (Fig. 2).

**Measurement of Absorption Spectra of DAC-R-Sepharose**—DAC-R-Sepharose for the determination of the absorption spectra was prepared as described by Miyagawa and Okuyama.<sup>10)</sup> A suitable amount of DAC-R-Sepharose was suspended in 0.05% agarose solution. A suspension of the same amount of R-Sepharose in 0.05% agarose solution was used as the control for this measurement. The amount of bound DAC was estimated from the molar absorption coefficient of colchicine at 350 nm (Fig. 2).

**Preparation of Lumicolchicine-R-Sepharose**—Lumicolchicine-R-Sepharose was prepared by the conversion

of DAC-R-Sepharose under irradiation with ultraviolet light (model EHB-W-300) as described by Morgan and Seeds.<sup>3)</sup> DAC-R-Sepharose was suspended in 95% ethanol and poured into a quartz tube of 15 mm diameter, then irradiated for 2 h in an ice bath. The conversion of DAC to lumicolchicine was confirmed by the characteristic absorption spectrum, having a weak maximum at 345 nm and a strong maximum at 265 nm (Fig. 3).<sup>11)</sup>

**Preparation of Bovine Brain 105000 × g Supernatant**—Bovine brain was homogenized with two volumes of 10 mM Pipes-NaOH buffer containing 1 mM guanosine-5'-triphosphate (GTP), 5 mM MgCl<sub>2</sub> and 10% glycerol, pH 6.9 (PGMG buffer). The homogenate was centrifuged at 105000 × g for 60 min in a Hitachi 80P-7 automatic preparative ultracentrifuge. The 105000 × g supernatant was used as the crude sample for affinity chromatography.

**Preparation of Tubulin**—Bovine brain tubulin was prepared by a two-cycle assembly-diassembly procedure according to the method of Berkowitz *et al.*,<sup>12)</sup> in 0.1 M Pipes-NaOH buffer containing 1 mM GTP, 2 mM ethylene glycol bis-(aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM MgCl<sub>2</sub> and 4 M glycerol, pH 6.9. The tubulin preparation was suspended in the same buffer and stored at -20 °C. The stored tubulin was diluted to 10 mM Pipes-NaOH buffer containing 1 mM GTP, 5 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub> and 10% glycerol, pH 6.9 (PCGMG buffer), and then applied to a column as the partially purified tubulin.

**Preparation of Tubulin-Colchicine Complex (TC Complex)**—TC complex was isolated by DEAE-Sephadex A-50 column chromatography according to the method of Arai and Okuyama,<sup>13)</sup> except for the use of 10 mM potassium phosphate buffer containing 20 μM colchicine, pH 7.0. For the experiment in Fig. 9, TC complex was dialyzed against colchicine-free 10 mM potassium phosphate buffer, pH 7.0, and used. On the other hand, TC complex used in affinity chromatography was prepared as follows. The reaction mixture of PCGMG buffer containing tubulin preparation (1.5 mg/ml) and 20 μM colchicine was incubated for 60 min at 37 °C with shaking, and the reaction mixture containing TC complex was applied to a column (Fig. 8).

**Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% slab gel according to the method of Laemmli.<sup>14)</sup> Slab gels were stained with Coomassie brilliant blue R-250. Micro two-dimensional polyacrylamide gel electrophoresis involving isoelectric focusing (first dimension) and electrophoresis on a 4–17% linear gradient gel containing 1% SDS (second dimension) was performed according to the method of Kadoya *et al.*<sup>15)</sup>

**Assay of Protein Content**—The protein content of samples was determined by the method of Lowry *et al.*,<sup>16)</sup> using bovine serum albumin as the standard substance.

## Results

### Chromatography of Two Types of Tubulin Preparations on DAC-R-Sepharose

Two types of tubulin preparations (crude and partially purified) were applied to the DAC-R-Sepharose. The 105000 × g supernatant obtained from bovine brain (extract from 5 g of brain) was applied to a column (0.5 i.d. × 3 cm) of DAC-succinyl-1,5-diaminopentane-Sepharose equilibrated with PGMG buffer. The elution profile is shown in Fig. 4A. The protein contents eluted with 0.1 M NaCl-PGMG buffer, with 6 M urea and 7 M guanidine-HCl were about 2.7, 3.5, and 1.7 mg protein/column, respectively. The eluates were analyzed by 0.1% SDS-PAGE (Fig. 5A). The eluate with 0.1 M NaCl-PGMG buffer contained a major component with an apparent molecular weight ( $M_r$ ) of 52000 daltons together with two minor components appearing at  $M_r$  43000 and 85000 daltons, respectively (Fig. 5A, lane 4). The content of the major component in this fraction was 70%. The mobility of the  $M_r$  52000 component was approximately the same as that of tubulin.

The micro two-dimensional gel electrophoresis showed that this major component had an isoelectric point ranging widely from 5.2 to 5.7 and consisted of two subunits ( $\alpha$ - and  $\beta$ -tubulin subunits) of different pI (Fig. 6B and C). Therefore, the major component of  $M_r$  52000 was thought to be tubulin, and a minor component of  $M_r$  43000 seemed to be actin protein. The second fraction obtained by 6 M urea elution contained several components other than the three components described above, and tubulin accounted for about 20% of the protein in the eluate (Fig. 5A-lane 5). The third fraction obtained by 7 M guanidine-HCl elution was essentially tubulin alone (Fig. 5, lane 6).

The biological activity of isolated tubulin was examined by colchicine-binding activity assay based on the method of Arai and Okuyama<sup>13)</sup> (data not shown). The tubulin, which was eluted by 0.1 M NaCl, showed a high activity and this activity was comparable to that of tubulin obtained by the assembly-disassembly method. The tubulin eluted with 6 M urea or

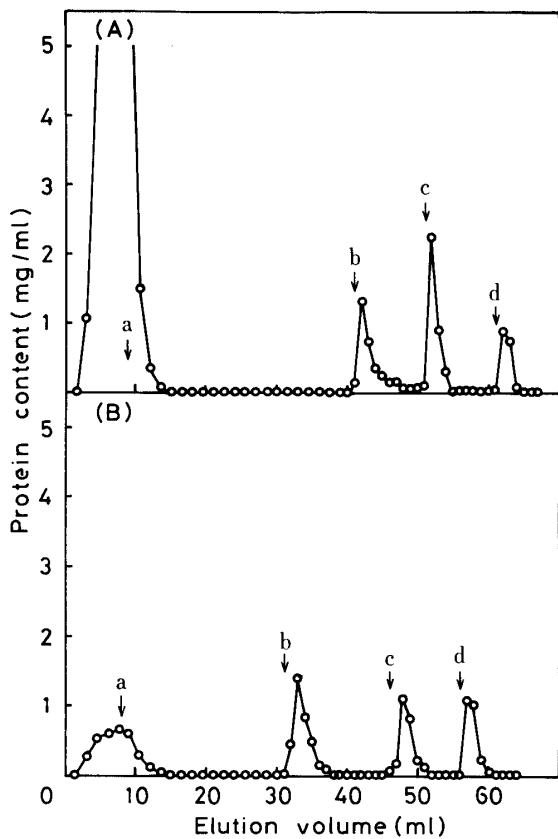


Fig. 4. Affinity Chromatography of Two Types of Tubulin Preparation on DAC-R-Sepharose

(A) The  $105000 \times g$  supernatant of bovine brain extract (5g brain) was applied to a column (0.5 i.d.  $\times$  3 cm) of DAC-succinyl-1,5-diaminopentane-Sepharose equilibrated with PGMG buffer. The flow rate was 4 ml/h. (a) Eluate with 30 ml of PGMG buffer was collected in fractions of 1.5 ml per tube. (b) Eluate with 15 ml of 0.1 M NaCl-PGMG buffer. (c) eluate with 10 ml of 6 M urea or (d) eluate with 10 ml of 7 M guanidine-HCl was collected in fractions of 1 ml per tube. (B) Tubulin preparation (15 mg protein/column) obtained by the assembly-disassembly method was applied to a column of the same adsorbent equilibrated with PCGMG buffer.

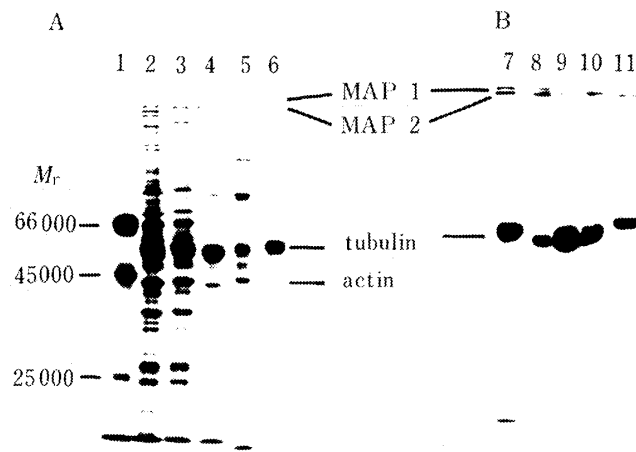


Fig. 5. Electrophoretic Analysis of Proteins Eluted from the Adsorbent

The fractions of eluates which showed rather high protein content in Fig. 4 were examined by 0.1% SDS-10% polyacrylamide gel electrophoresis. (A) Lane 1; bovine serum albumin ( $M_r$  66000), ovalbumin ( $M_r$  45000) and chymotrypsinogen A ( $M_r$  25000). Lane 2; the  $105000 \times g$  supernatant before the affinity chromatography. Lane 3; the passed through fraction of lane 2. Lane 4; the eluate with 0.1 M NaCl-PGMG buffer. Lane 5; the eluate with 6 M urea. Lane 6; the eluate with 7 M guanidine-HCl. (B) Lane 7; tubulin preparation obtained by the assembly-disassembly method. Lane 8; the passed through fraction of tubulin preparation. Lane 9; the eluate with 0.1 M NaCl-PCGMG buffer. Lane 10; the eluate with 6 M urea. Lane 11; the eluate with 7 M guanidine-HCl.

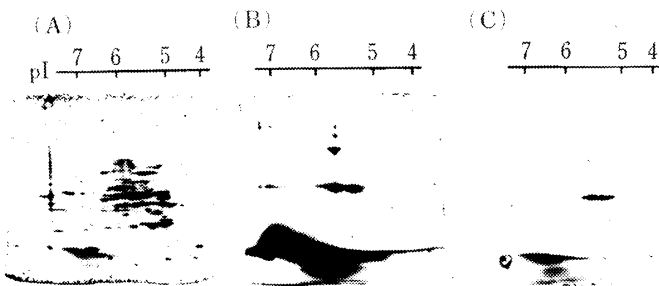


Fig. 6. Micro Two-Dimensional Gel Electrophoresis of Tubulin Purified by Affinity Chromatography

(A) The  $105000 \times g$  supernatant (2  $\mu$ l), (B) partially purified tubulin preparation (5  $\mu$ g protein), (C) the eluate with 0.1 M NaCl-PCGMG buffer (4  $\mu$ l) shown in Fig. 4A.

7 M guanidine-HCl did not show colchicine-binding activity.

Using a tubulin preparation (about 15 mg protein) obtained from bovine brain by the assembly-disassembly method, a similar experiment was performed on a column (0.5

TABLE I. Rechromatography of the Passed-through Fraction and the 0.1 M NaCl Fraction on a DAC-CH-Sepharose Column

	Amount of tubulin charged on column	The passed-through fraction (mg protein/column)	Adsorption capacity		
			0.1 M NaCl	6 M urea	7 M guanidine-HCl
First column	60	27	20	3	6
Second column					
The passed-through fraction	27	10	12	2	3
The 0.1 M NaCl fraction	20	1	14	2	4

Tubulin (about 60 mg protein) obtained by the assembly-disassembly method was applied to the first column (DAC-carboxyhexyl-Sepharose; 1.0 cm i.d.  $\times$  3 cm) equilibrated with PCGMG buffer. The flow rate was 15 ml/h (5 ml/tube). The column was washed with PCGMG buffer for 4 h, and the adsorbed tubulin was eluted with 0.1 M NaCl, 6 M urea and 7 M guanidine-HCl. The passed through fraction or the 0.1 M NaCl fraction obtained from the first column chromatography was dialyzed for 6 h against PCGMG buffer and applied to the second column under the same conditions as used for the first column.

TABLE II. Effect of Spacers on Affinity between Immobilized Colchicine and Tubulin

Adsorbents <sup>a)</sup>	Ligand content ( $\mu$ mol/g wet weight of adsorbent)	Spacer length ( $\text{\AA}$ )	Adsorption capacity <sup>b)</sup> (mg protein/g wet weight of adsorbent)	
			105000 $\times$ g supernatant	Tubulin
DAC-Sepharose	1.50		6.1	5.6
Group A DAC-OC(CH <sub>2</sub> ) <sub>n</sub> NH-Sepharose				
A-1	1.50	5.0	6.3	4.8
A-2	1.54	7.5	30.2	25.6
Group B DAC-OC(CH <sub>2</sub> ) <sub>2</sub> CONH(CH <sub>2</sub> ) <sub>n</sub> NH(CH <sub>2</sub> ) <sub>n</sub> NH-Sepharose				
B-1	1.53	12.1	9.2	8.1
B-2	1.52	14.7	12.5	10.8
Group C DAC-OC(CH <sub>2</sub> ) <sub>2</sub> CONH(CH <sub>2</sub> ) <sub>n</sub> NH-Sepharose				
C-1	1.58	9.8	8.3	4.7
C-2	1.54	11.0	10.7	8.1
C-3	1.62	12.2	22.3	20.4
C-4	1.52	13.6	19.7	22.9
C-5	1.58	14.8	22.9	25.6
C-6	1.56	16.0	18.7	17.5
C-7	1.55	17.2	31.8	26.4

a) The classification of adsorbents is shown in Fig. 1. b) The value represents the total capacity of 0.1 M NaCl and 6 M urea eluates in the affinity chromatography shown in Fig. 4.

i.d.  $\times$  3 cm) of DAC-succinyl-1,5-diaminopentane-Sepharose equilibrated with PCGMG buffer. Its elution profile is shown in Fig. 4B. Using the three elution buffers (3.5, 2.5 and 2.4 mg protein/column), adsorbed tubulin was successively eluted but 4.8 mg of tubulin passed through the column. The tubulin recovery was about 88%, and the adsorbed tubulin was about 33.6 mg protein/g wet weight of adsorbent. The tubulin preparation obtained by the assembly-disassembly method contained microtubule-associated proteins (MAP-1 and -2 protein) other than tubulin (Fig. 5B, lane 7). The electrophoresis analysis of three eluates showed a band corresponding to the migration position of MAP-2 protein rather than MAP-1

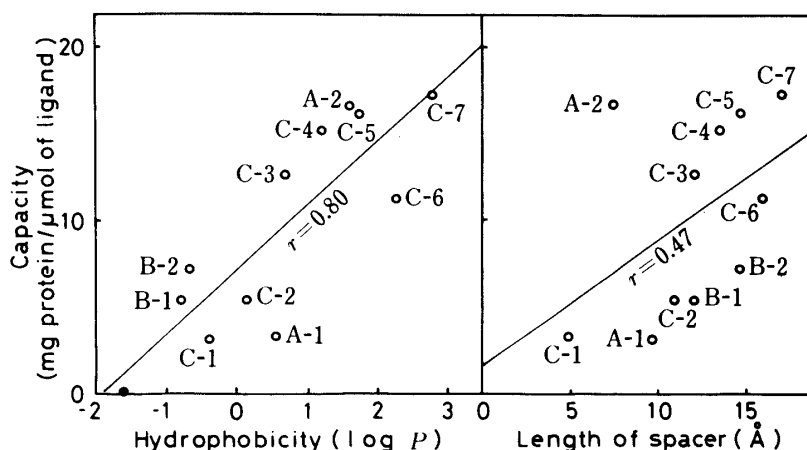


Fig. 7. Effect of the Hydrophobicity and the Length of Spacer on the Adsorption Capacity for Tubulin

The adsorbed quantity of tubulin per micro mole of colchicine ligand was calculated based on the data in Table I. The hydrophobicity ( $\log P$ ) of the spacer with the colchicine ligand was calculated by means of the equation  $\log P = \sum_1^n a_n f_n + c$  based on Rekker's hydrophobic fragmental constant ( $f$ ).<sup>17)</sup> The numbers of the open circles correspond to the adsorbents shown in Table I, and the closed circle shows the  $\log P$  of succinyl-1,5-diaminopentane-Sepharose without colchicine ligand (Table II);  $r$  is the correlation coefficient.

protein (Fig. 5B, lanes 9, 10 and 11).

Further, from the elution profile of tubulin, there were at least three binding types of tubulin: the first passes easily through the column, the second is eluted by 0.1 M NaCl-PCGMG buffer with a weak ionic strength, and the third is eluted by strong denaturants such as 6 M urea and 7 M guanidine-HCl.

To examine the reproducibility of the binding types, rechromatography of the passed-through fraction and the 0.1 M NaCl eluted fraction was carried out (Table I).

#### Effect of Spacers on the Affinity between Tubulin and Immobilized Colchicine

In order to examine the effect of spacers on the affinity between tubulin and immobilized colchicine, eleven kinds of DAC-R-Sepharose with various spacers were synthesized as shown in Fig. 1. Their adsorption capacities for tubulin are summarized in Table II. The adsorption capacity of DAC-Sepharose without any spacer was 6.1 or 5.6 mg protein/g wet weight of adsorbent using the 105000  $\times g$  supernatant or the tubulin preparation obtained by the assembly-diassembly method, respectively. In comparison with this, the capacities of DAC-R-Sepharose were large, and the adsorption capacity showed a tendency to increase in proportion to the length of the spacer in group A, B or C. Comparing adsorbents B-1 and C-3 having the same spacer length, the adsorption capacity of adsorbent C-3 was about 2.5 times greater than that of adsorbent B-1. The difference in spacer structures (as shown in Fig. 1) was only one imino or methylene group, but the insertion of an imino group markedly reduced the hydrophobicity of the spacer. A similar result was found with adsorbents B-2 and C-5. Further, the adsorption capacity of adsorbents C-1 and C-2, which had a longer spacer but lower hydrophobicity than adsorbent A-2, was about one-third of that of adsorbent A-2. Adsorbents which showed a marked increase in adsorption capacity were A-2, C-3, C-4, C-5, C-6 and C-7. These six adsorbents had a common structure containing a continuous methylene chain of more than four units in the spacer. Therefore, the effect of the spacer on the adsorbed quantity of tubulin was examined from the view point of the hydrophobicity and the length of the spacer (Fig. 7). The correlation coefficient between the hydrophobicity of spacer and the adsorbed quantity of tubulin was 0.80, whereas the correlation coefficient

between the length of spacer and the adsorbed quantity of tubulin was 0.47. This result indicates that the effect of the spacer on the tubulin-immobilized colchicine interaction depended on the hydrophobicity rather than the length of the spacer.

### Effects of Changes of Hydrophobic Parameters on the Adsorption Capacity and the Binding Types

It is generally known that the hydrophobic adsorption is affected by ionic strength,<sup>18a-c)</sup> temperature,<sup>18a)</sup> polyhydroxyl compounds<sup>18c,19)</sup> and surface-active agents.<sup>20)</sup> When tubulin was applied to DAC-CH-Sepharose equilibrated with PCGMG buffer at 4 °C, the adsorption capacity was about 30 mg of protein and the amounts of tubulin eluted with 0.1 M NaCl, 6 M urea and 7 M guanidine-HCl were 25, 3 and 3 mg protein, respectively (Table III). The adsorption capacity and the elution pattern did not change at 25 °C. However, when the concentration of glycerol of PCGMG buffer was raised to 40%, the adsorption capacity did not change, but the amount of tightly bound tubulin which was eluted by 7 M guanidine-HCl increased. Moreover, in the presence of 1 M NaCl, the adsorption capacity decreased, but the amount of tightly bound tubulin markedly increased.

On the other hand, the adsorption capacity and the distribution on aminohexyl-Sepharose was not affected by an increase of glycerol (Table III). However, in the presence of 1 M NaCl, tubulin was not adsorbed on AH-Sepharose.

### Effects of Various Ligands on the Adsorption Capacity to Tubulin

As shown in Table IV, tubulin was not adsorbed on the matrix itself (Sepharose 4B) or on the adsorbents with a carboxyhexyl group (No. 2) or a succinylaminohexyl group (No. 4). In contrast, the adsorbent with aminohexyl group (No. 3) showed a good adsorption capacity for tubulin.

It is known that colchicine inhibits cell division by binding to tubulin, but lumicolchicine (formed by a rearrangement of ring C of colchicine) does not. Using immobilized lumicolchicine as the adsorbent, the specificity of the ligands for tubulin was examined (No. 6). The adsorption capacity of immobilized lumicolchicine for tubulin was not different from that of immobilized colchicine (No. 5).

Commercial phenyl-Sepharose (No. 7), which is used as an adsorbent in hydrophobic chromatography, did not adsorb tubulin, whereas phenyl-AH-Sepharose (No. 8) with the aminohexyl spacer showed a good adsorption. Moreover, the hydrophobic adsorbents with ligands such as the trimethoxyphenyl group (No. 9), diphenyl group (No. 10) and naphthyl

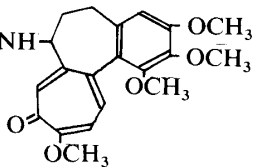
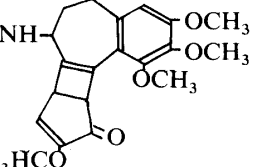


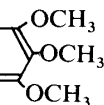
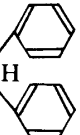
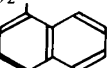
TABLE III. Effects of Changes of Hydrophobic Parameters on the Adsorption Capacity and the Binding Types

Adsorbent	Conditions of equilibrating buffer <sup>b)</sup>			Adsorption capacity (mg protein/g wet weight of adsorbent)		
	Temp. (°C)	Glycerol (%)	NaCl (M)	0.1 M NaCl	6 M urea	7 M guanidine-HCl
DAC-CH-Sepharose <sup>a)</sup>	4	10	0	25	3	3
DAC-CH-Sepharose	25	10	0	27	3	2
DAC-CH-Sepharose	4	40	0	18	N.D.	15
DAC-CH-Sepharose	4	10	1	0	N.D.	20
AH-Sepharose <sup>a)</sup>	4	10	0	16	5	10
AH-Sepharose	4	40	0	18	N.D.	10
AH-Sepharose	4	10	1	0	N.D.	0

a) Tubulin (50 mg protein) obtained by the assembly-disassembly method was applied to a column (1.0 cm i.d. × 3 cm) equilibrated with PCGMG buffer as shown in Fig. 4. b) PCGMG buffer conditions as the standard. The column chromatography was carried out by the procedure shown in Table I.



TABLE IV. Specificity of Ligand Species

Adsorbent	Adsorption capacity (mg protein/g wet weight of adsorbent)		
	0.1 M NaCl	6 M urea	7 M guanidine-HCl
1 (Sephacrose 4B) $\ddagger$	0	0	0
2 $\ddagger$ NH(CH <sub>2</sub> ) <sub>5</sub> COOH	0	0	0
3 $\ddagger$ NH(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub>	16	5	10
4 $\ddagger$ NH(CH <sub>2</sub> ) <sub>6</sub> NHCO(CH <sub>2</sub> ) <sub>2</sub> COOH	0	0	0
5 $\ddagger$ NH(CH <sub>2</sub> ) <sub>5</sub> CONH- 	25	3	3
6 $\ddagger$ NH(CH <sub>2</sub> ) <sub>5</sub> CONH- 	24	3	2
7 $\ddagger$ OCH <sub>2</sub> CH(OH)CH <sub>2</sub> -O- 	2	6	0
8 $\ddagger$ NH(CH <sub>2</sub> ) <sub>6</sub> NHCO- 	20	3	4
9 $\ddagger$ NH(CH <sub>2</sub> ) <sub>6</sub> NHCOCH=CH- 	3	2	20
10 $\ddagger$ NH(CH <sub>2</sub> ) <sub>5</sub> CONH(CH <sub>2</sub> ) <sub>2</sub> CH- 	12	3	13
11 $\ddagger$ NH(CH <sub>2</sub> ) <sub>5</sub> CONH(CH <sub>2</sub> ) <sub>2</sub> NH- 	25	3	2

The conditions of column chromatography were the same as in Table I.

group (No. 11) also adsorbed tubulin. The adsorbed tubulin on immobilized trimethoxyphenyl, which consists of ring A in the colchicine structure, was mostly eluted with 7 M guanidine-HCl.

#### Binding of TC Complex to DAC-R-Sephacrose

It is believed that 1 mol of colchicine is bound to 1 mol of tubulin, so that the TC complex would not be adsorbed on immobilized colchicine. TC complex was applied to DAC-succinyl-1,5-diaminopentane-Sephacrose, and elution was performed as shown in Fig. 8 (A and B). Most of the protein bound to the colchicine column and was eluted with 0.1 M NaCl-PCGMG buffer, and a small amount was eluted with 6 M urea. The fluorescence intensity of the major peak was proportional to the protein content. In Fig. 8B, the control experiment with tubulin without colchicine is shown. This result may indicate that the binding mode of TC complex to colchicine column differs from that of colchicine to tubulin in the TC complex.

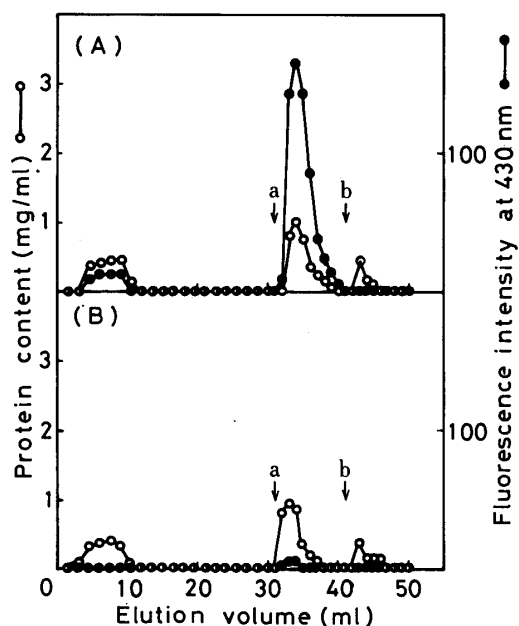


Fig. 8. Elution Profile of TC Complex on DAC-succinyl-1,5-diaminopentane-Sepharose

(A) TC complex (10 mg protein/column) was applied to DAC-succinyl-1,5-diaminopentane-Sepharose equilibrated with PCGMG buffer. (B) Tubulin (10 mg protein/column) obtained by the assembly-disassembly method was applied to the same adsorbent. The arrows a and b represent the position at which the elution buffer was changed (0.1 M NaCl-PCGMG buffer and 6 M urea). TC complex was prepared by incubation of the tubulin preparation with shaking for 60 min at 37°C in PCGMG buffer and its colchicine-binding activity was determined according to the method of Arai and Okuyama.<sup>13)</sup>

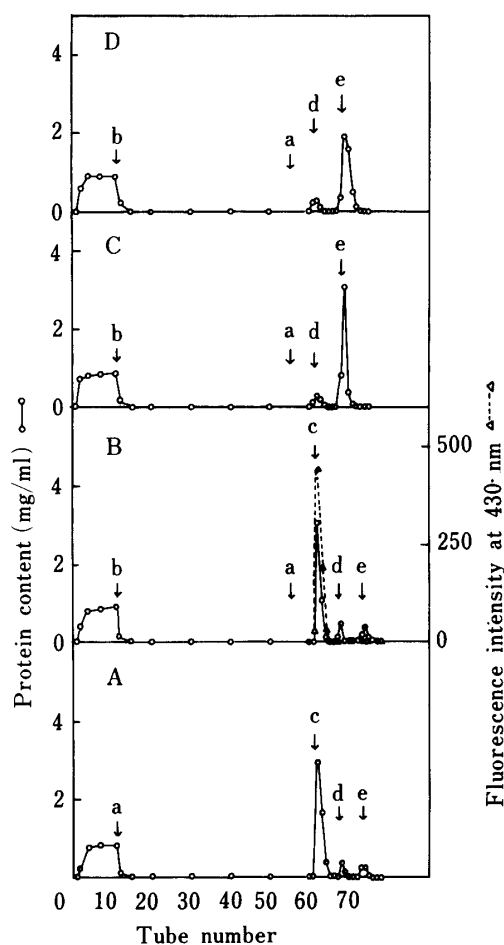


Fig. 9. Change of Binding Mode between Tubulin and Immobilized Colchicine

The adsorbents in A to C and D were DAC-CH-Sepharose and AH-Sepharose, respectively. The column size was 1.0 cm i.d.  $\times$  3 cm and the flow rate was 15 ml/h (5 ml/tube). Tubulin (about 60 mg protein) obtained by the assembly-disassembly method was applied to the column equilibrated with PCGMG buffer. The arrows represented the positions at which the buffer was changed: a, PCGMG buffer; b, PCGMG buffer containing 50  $\mu$ M colchicine; c, 0.1 M NaCl-PCGMG buffer; d, 6 M urea; e, 7 M guanidine-HCl. The colchicine-binding activity was expressed in terms of the fluorescence intensity at 430 nm (excitation at 350 nm).

### Change of the Binding Mode between Tubulin and Immobilized Colchicine

As shown in Fig. 4, a part of the adsorbed tubulin could be removed with 0.1 M NaCl, whereas the remaining tubulin was bound more tightly and required 7 M guanidine-HCl for elution. This pattern was independent of the ligand concentration but was dependent on the length of time that the tubulin remained on the column.<sup>5)</sup> After washing of the column for 16 h with PCGMG buffer, the elution profile of the adsorbed tubulin was unchanged in comparison with that in the case of the washing for 4 h (Table I), and about 85% of the adsorbed tubulin was eluted with 0.1 M NaCl (Fig. 9A). A time-dependent increase of tightly bound tubulin on the column was not seen.

When the column with adsorbed tubulin was washed with PCGMG buffer containing 50  $\mu$ M colchicine, the tubulin eluted with 0.1 M NaCl exhibited a high colchicine-binding activity, and this result indicated that free colchicine bound to the adsorbed tubulin on

immobilized colchicine (Fig. 9B).

When the adsorbed tubulin was eluted with 6 M urea as the first buffer, the amount of eluted tubulin was about 10%, and the remaining tubulin was eluted with 7 M guanidine-HCl (Fig. 9C). Further, the same experiment on the adsorbent AH-Sepharose gave the same result as obtained on DAC-CH-Sepharose (Fig. 9D). These results indicate that tight binding of tubulin is closely related to the spacer.

### Discussion

The usefulness of hydrophobic chromatography for the fractionation of biological materials has been reported and some general aspects of the method were discussed.<sup>21)</sup> Most adsorbents in use have been synthesized by the cyanogen bromide procedure, which is useful to introduce positively charged groups into the adsorbent matrix.<sup>22)</sup> Therefore, such gel materials exhibit ion-exchange properties superimposed upon the hydrophobic interaction. Regarding the charge properties of monoaminoalkanes attached to Sepharose, it was reported that this type of adsorbent was positively charged at near-neutral pH and had the  $pK_a$  value of 9.6.<sup>23)</sup> The carboxy groups of succinylaminoalkyl-Sepharose, introduced by the treatment with succinic anhydride, had the  $pK_a$  value of about 4.5.<sup>23)</sup> Moreover, since the  $pK_a$  value of the coupling isourea groups was 9.6, this adsorbent with a carboxy-end had approximately equal numbers of anionic and cationic groups.<sup>23)</sup> Therefore, it is suggested that the succinylaminoalkyl-Sepharose shows a weaker ion-exchange ability than aminoalkyl-Sepharose.

Since the isoelectric point of tubulin is approximately 5.5, tubulin is a negatively charged protein at pH 6.9. Therefore, it is suggested that the adsorption of tubulin on aminohexyl-Sepharose is due to ion-exchange interaction, and the desorption of tubulin from carboxyhexyl- and succinylaminohexyl-Sepharose is a result of repulsion between tubulin and carboxy-end groups (Table IV). Moreover, in the presence of 1 M NaCl, tubulin was not adsorbed on AH-Sepharose (Table III), so this observation indicated that the binding of tubulin and immobilized aminohexyl groups was due to electrostatic force rather than hydrophobic force. On the other hand, the use of 6 M urea as the first elution buffer increased the amount of tightly bound tubulin which was eluted with 7 M guanidine-HCl (Fig. 9D). These observations suggested that the binding between tubulin and immobilized aminohexyl groups is at least a two-step reaction. Namely, the first step is a reversible binding based on the electrostatic force and the second step is an irreversible binding based on the hydrophobic interaction between the hexamethylene moiety in the aminoalkyl group and tubulin accompanied with conformational change.

The adsorbents with hydrophobic ligands such as phenyl, diphenyl, trimethoxyphenyl and naphthyl group have a lower positive charge than AH-Sepharose but show a high adsorption capacity for tubulin (Table IV). However, phenyl-AH-Sepharose, with a weak positive charge of the coupling isourea group, showed the ability to adsorb tubulin, whereas commercial phenyl-Sepharose (without positive charge) did not (Table IV). This observation may indicate that the hydrophobic DAC-R-Sepharose adsorbents bind tubulin through both hydrophobic and electrostatic forces. On the other hand, in the presence of 1 M NaCl, the adsorption capacity of DAC-CH-Sepharose was about 65% of the standard amount, but the adsorbed tubulin mostly showed irreversible binding, being eluted with 7 M guanidine-HCl (Table III). Furthermore, the treatment with 6 M urea caused an increase of irreversibly bound tubulin (Fig. 9C). These observations suggested that the binding between tubulin and immobilized colchicine was at least a two-step reaction. Namely, the first step is a reversible binding based on cooperation between the hydrophobic and the electrostatic forces. However, the electrostatic force of DAC-CH-Sepharose is very much weaker than that of AH-Sepharose, so

that reversible binding of immobilized colchicine may result from the hydrophobic force rather than the electrostatic force. The second step is an irreversible binding based on the hydrophobic interaction between methylene chains in the spacer and tubulin, accompanied with conformational change. It is suggested that this reaction proceeds successively but the second step may occur independently in the presence of 1 M NaCl or 6 M urea. Therefore, the binding mode of DAC-R-Sepharose differs from that of AH-Sepharose.

The binding site of tubulin to immobilized colchicine was not located at the active center for colchicine-binding activity (Fig. 9B) but in the hydrophobic region (Table IV). These observations suggest that the binding of tubulin is a nonspecific adsorption owing to the hydrophobic force. A better correlation of the tubulin-adsorbing capacity with the hydrophobicity of the spacer was obtained (Fig. 7). Furthermore, the adsorbents having a high adsorption capacity for tubulin had a common structure with a continuous methylene chain of more than four units in the spacer.

Finally, the binding between tubulin and immobilized colchicine is not a specific adsorption but a non-specific one. Therefore, DAC-R-Sepharose can not be used for the specific isolation of tubulin, because of the adsorption of other proteins (Fig. 5). However, the application of this adsorbent to hydrophobic chromatography might be interesting. Recently, in a preliminary experiment, two proteins with apparent molecular weight of 35000 and 85000 showed good adsorption of DAC-CH-Sepharose after tubulin had been removed by ammonium sulfate fractionation. Furthermore, it is thought that DAC-R-Sepharose may be applicable to the isolation of various proteins by modification of the hydrophobic parameters. At present, we are studying the application of these adsorbents to hydrophobic chromatography.

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