Purification of Yeast Tubulin by Self-Assembly in Vitro†

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ABSTRACT: Tubulin was purified from yeast homogenate by DEAE-Sephadex column chromatography and temperature-dependent assembly. The yeast tubulin subunits comigrate with the brain α-tubulin subunit on one-dimensional sodium dodecyl sulfate gel electrophoresis. The in vitro yeast tubulin assembly is inhibited by the fungicide methyl *N*-(benzimidazol-2-yl)carbamate, the active component of benomyl, whereas in vitro brain 6S tubulin assembly is resistant. This suggests that the inhibitory effect of benomyl on yeast cell division is due to its antimicrotubule action.

Yeast and other Ascomycete fungi have considerable potential for investigating the biochemistry and function of microtubules and other filamentous proteins. They can be grown cheaply in large quantities, and in the case of yeast under synchronous conditions. Methods are available for their fractionation, including the isolation of nuclei (Rozijn & Tonino, 1964). Perhaps of greatest importance in these studies is the ease of genetic manipulation in the Ascomycetes: a large number of cell division mutants are already available (Hartwell, 1974; Nurse et al., 1976; Morris, 1980) and others can be obtained; mutants resistant to antimicrotubular agents which have defects in the β-tubulin subunits have been obtained and analyzed (Sheir-Neiss et al., 1978). The potential of the Ascomycetes for establishing the function of filamentous proteins is already starting to be realized in studies on nuclear migration in Aspergillus (Oakley & Morris, 1980). Most recently the transformation of yeast by a yeast gene clone in a bacterial plasmid vector (Hinnen et al., 1978) has increased the possibilities for manipulation of specific genes in yeast. Already the wild-type *his*3 gene of yeast has been replaced in the correct chromosomal position by a cloned mutant *his*3 gene containing an internal deletion (Scherer & Davis, 1979). Mutants with deleted tRNA genes have also been produced (Wallace et al., 1980). The recent cloning of the α- and β-tubulin genes (Cleveland et al., 1980) raises the possibility of the production of a large number of yeast tubulin mutants by these methods. Before using these mutants defective in microtubule assembly, it is necessary to obtain tubulin from yeast, to assemble it into microtubules in vitro, and to demonstrate some of the characteristics of the assembly process, including those related to fungicides known to inhibit mitosis in vivo (Davidse, 1973). A previous attempt to assemble Aspergillus tubulin from an enriched DEAE-Sephadex fraction was unsuccessful (Davidse & Flach, 1977), though recently Physarum tubulin has been successfully assembled without DEAE-Sephadex enrichment (Roobol et al., 1980).

This paper reports the purification of yeast tubulin by enrichment on a DEAE-Sephadex column (Weisenberg et al., 1968) followed by temperature-dependent self-assembly. It is demonstrated that benzimidazole fungicides which have been proposed to inhibit fungal growth by blocking microtubule assembly in vivo (Davidse, 1973) are highly effective inhibitors of yeast tubulin assembly in vitro.

Experimental Procedures

Purification of Yeast Tubulin. Saccharomyces uvarum (NCYC 74) or *S. cerevisiae* (SC-3) was grown in 9 L of 3 g/L malt extract, 10 g/L glucose, 3 g/L yeast extract, and 5 g/L bactopeptone to late log phase (3×10^7 cells/mL) at 25 °C under vigorous aeration. In the case of *S. uvarum* this yielded 50 g wet weight of cells which were treated with mercaptoethanol (Klein & Byers, 1978) and permeabilized with 6 mL of snail digestive juice (HPJ, Uniscience, Cambridge, U.K.) and 15 mg of Zymolyase 5000 (Kirin Brewery, Japan) for 2 h at 30 °C. After being washed with 1.1 M sorbitol the cells were homogenized in 0.1 M Pipes-Na, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA,[1] 1 mM dithiothreitol, 1 mM GTP (Li salt), and 1:100 solution P (87 mg of *p*-methylphenylsulfonyl fluoride and 1.5 mg of pepstatin A in 5 mL of absolute ethanol). The homogenate was centrifuged at 100000*g* for 45 min and the supernatant (1100 mg of protein) made 0.2 M in KCl and applied to a 5 cm diameter × 3.5 cm long column of DEAE-Sephadex A-50 equilibrated with 0.1 M Pipes-Na, pH 6.9, 0.2 M KCl, 0.2 mM $MgCl_2$, 0.1 mM GTP, and 1:1000 solution P. After being washed with 350 mL of buffer the enriched tubulin was eluted by raising the KCl to 0.5 M and concentrated (Amincon 400, PM-10 membrane) to about 4 mL. Ammonium sulfate (310 mg/mL) was added, the pellet dissolved in column buffer without KCl, and gel filtered against the same buffer. The sample was concentrated to about 0.5 mL (around 30 mg/mL), and GTP was added to 1 mM and solution P to 1:100. Bundles of microtubules were assembled (monitored by dark-field light microscopy) by incubation at 25 °C for 5 min and pelleted at 30 000 rpm in a Beckman type 65 rotor for 5 min at 25 °C. Speed is essential at this step since the microtubules are unstable; incubation and centrifugation should take less than 20 min. The pellets were extracted with 40 μL of 0.1 M Pipes-Na, pH 6.9, 0.1 mM $MgCl_2$, 1 mM GTP, and 1:100 solution P for 30 min at 4 °C and centrifuged at 50000*g* for 5 min at 4 °C. The supernatant (containing about 300 μg of protein) could be assembled again into microtubules at 25 °C (these microtubules were very stable) or stored at −80 °C without loss of activity over several months. The pellets were also stored at −80 °C and could be cold extracted up to 4 times to give polymerizable tubulin; yeast microtubules break down very slowly in the cold. The total yield of tubulin was about 0.5 mg, corresponding to 0.05% of the soluble protein.

Coassembly of ^{35}S-Labeled Yeast Tubulin with Brain Tubulin. S. uvarum (NCYC 74) was grown to 2×10^7 cells/mL in the ^{35}S medium of Baum et al. (1978). Cells were resus-

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[1] Abbreviations used: MBC, methyl *N*-(benzimidazol-2-yl)carbamate; MAP, microtubule-associated protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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pended in 0.1 M Pipes-Na, pH 6.9, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM $MgCl_2$, 1 mM GTP, and 1:100 by volume solution P and broken open by vortexing with glass beads. After centrifugation at 100000*g* for 60 min at 4 °C an equal volume of pig brain tubulin (7 mg/mL), prepared as described by Borisy et al. (1975), was added and at least two cycles of temperature-dependent assembly–disassembly carried out. The ^{35}S-labeled DEAE-Sephadex fraction was prepared as described earlier for nonradioactive yeast and cycled up to 2 times with brain tubulin as above.

Gel Electrophoresis. $NaDodSO_4$ gels were run as described by Sheir-Neiss et al. (1978) by using the specially purified grade of $NaDodSO_4$ from BDH. Fluorography was carried out as described by Bonner & Laskey (1974) and Laskey & Mills (1975). Two-dimensional gels were run as described by O'Farrell (1975) except that the second dimension was the $NaDodSO_4$ system of Sheir-Neiss et al. (1978).

Dark-Field Light Microscopy. Yeast microtubules were detected by dark-field light microscopy with an Osram 150-W xenon lamp (XBO 150W/1) mounted on a standard Zeiss microscope. The dark-field condenser was a Zeiss ultracondenser, N.A. 1.2/1.4, and the objective a Zeiss neofluar, 40X, N.A. 0.75.

Measurement of the Effect of Benzimidazoles and Colchicine on Yeast and Brain Tubulin Assembly by Quantitative Electron Microscopy. A modification of the method of quantitative electron microscopy (Kirschner et al., 1975) was used to measure the effects of benzimidazoles and colchicine on tubulin assembly. Bushy stunt virus was replaced by tobacco mosaic virus; its long rods were much easier to see in the droplets than the small spherical bushy stunt particles. The uranyl acetate stain was replaced after fixation by neutral phosphotungstate; this made the microtubules more visible.

The stock solution of yeast tubulin (purified by one cycle of assembly–disassembly) was centrifuged just beforehand at 40000*g* and 4 °C to remove all cold-stable microtubules. Associated proteins were removed from brain tubulin by chromatography on Bio-Gel Al.5m (Sloboda et al., 1976). MBC and nocodazole were dissolved in dimethyl sulfoxide and colchicine in water and added to 3 μL of tubulin so that total dimethyl sulfoxide was 5% for MBC and 2% for nocodazole. The samples in 0.1 M Pipes-Na, pH 6.9, 0.1 mM $MgCl_2$, and 1 mM GTP were incubated for 30 min in ice, then at 25 °C for 20 min for yeast tubulin (2–4 mg/mL), and at 37 °C for 5 min for brain 6S tubulin (2–4 mg/mL). For each inhibitor, the protein concentrations in a control were adjusted until about the same amount of assembly was found as judged by dark-field light microscopy. After incubation, 0.15 μL of 25% glutaraldehyde was added followed by 1 μL of tobacco mosaic virus (1 mg/mL, supplied by P.J.G. Butler). After fixation for 10 min nonvolatile salts were exchanged for volatile ones by addition of two beads of Amberlite IRA-400 (CH_3COO^- form) and two beads of Amberlite IR-120 (NH_4^+ form). After 3 min the sample was removed and mixed with an equal volume of 40 mg/mL sodium phosphotungstate (pH 7.0) in 4 mg/mL sucrose, and 0.5 μL was sprayed onto a carbon-coated glow discharged grid with the E. F. Fullam spray gun (no. 1180). The microtubule concentration was calculated from the total length of microtubules divided by the total length of tobacco mosaic virus rods in a particular drop and plotted against the fungicide or drug concentration.

Results

Purification of Yeast Tubulin. Yeast tubulin was partially purified from a high-speed supernatant of yeast cell homogenate by using DEAE-Sephadex chromatography (Weisenberg et al., 1968); it was assayed by coassembly of the eluted ^{35}S-labeled yeast fractions with brain tubulin followed by $NaDodSO_4$ gel electrophoresis of the assembled microtubules to identify radioactive bands with the approximate mobility of tubulin. When the elution conditions had been defined, the procedure was scaled up with nonradioactive yeast without carrier brain tubulin. When the concentrated material (Figure 1, lane 2) was incubated at 25 °C, bundles of yeast microtubules assembled as observed by dark-field light microscopy. Thin sections of a tannic acid stained pellet (Kim et al., 1979) showed that the microtubules had mainly 12 protofilaments (70% 12 protofilaments, 30% 13 protofilaments; data not shown). $NaDodSO_4$ gel electrophoresis of this pellet (Figure 1, lane 4) showed enrichment for protein with mobility slightly less than that of brain tubulin (Figure 1, lanes 1 and 7). A second cycle of temperature-dependent disassembly–assembly further enriched for the protein (Figure 1, lane 6) and a tannic acid stained thin section of this pellet (Figure 2) showed exclusively microtubules, thus identifying the protein as tubulin. These microtubules contained predominantly 13 protofilaments (90% 13 protofilaments, 10% 12 protofilaments).

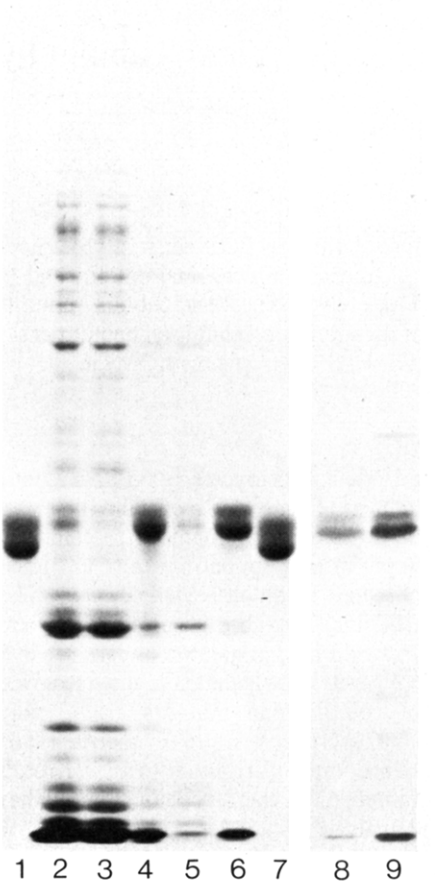

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of fractions during the purification of yeast tubulin. Lanes 1–7 are from a Coomassie blue stained gel; lanes 8 and 9 are from a fluorogram of the coassembly of ^{35}S yeast proteins with brain tubulin. Lanes 1 and 7 are brain 6S tubulin (Sloboda et al., 1976). Lane 2 is yeast homogenate after enrichment for yeast tubulin on a DEAE–Sephadex column; lane 3 is the supernatant; lane 4 the pellet after incubation of the DEAE-Sephadex fraction at 25 °C to form yeast microtubules. This pellet was solubilized at 4 °C and a second cycle of assembly at 25 °C is shown in lanes 5 (supernatant) and 6 (pellet). Lane 8 is the ^{35}S yeast DEAE-Sephadex fraction after coassembly with brain tubulin and lane 9 the ^{35}S yeast cell homogenate, also after coassembly with brain tubulin.

It should be noted that there were no obvious high molecular weight microtubule-associated proteins (MAP's) copurifying

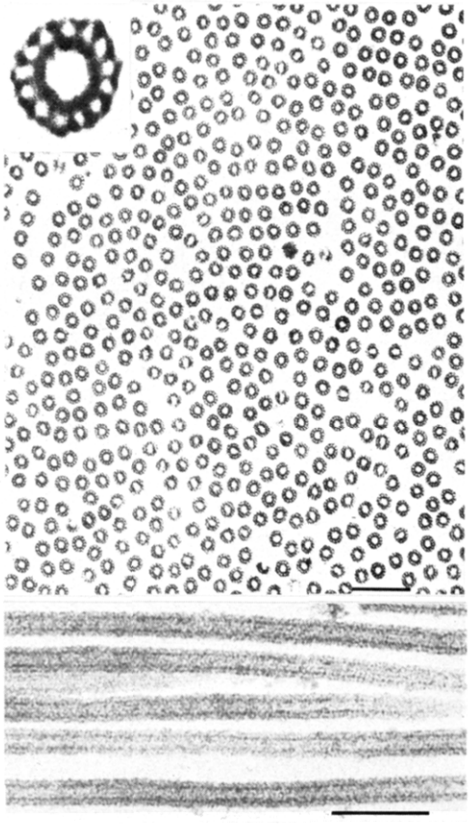

FIGURE 2: Thin sections of a tannic acid stained pellet of yeast microtubules after two cycles of assembly–disassembly. Top: cross section; the insert shows a magnified view of one microtubule. Bottom: longitudinal section. Bar 0.1 μm.

with the in vitro assembled yeast tubulin, though the absence of low molecular weight MAP's (M_r <20 000) cannot be excluded. However if they had similar charge properties to brain MAP's then the DEAE-Sephadex purification would have removed them (Murphy & Borisy, 1975).

The yeast tubulin partially purified by the DEAE-Sephadex column coassembled with brain tubulin (Figure 1, lane 8) and had a mobility identical with that of the coassembled product from whole yeast homogenate (Figure 1, lane 9). This indicates that the yeast tubulin enriched by the DEAE-Sephadex procedure is similar to the whole cell tubulin.

Yeast tubulin, like brain tubulin, contains two major subunits, as can be seen from a comparison of the same area of two-dimensional gels (Figure 3). The principal basic polypeptide on the two-dimensional gel of yeast tubulin presumably corresponds to the α-tubulin of brain and the principal acidic polypeptide to the β-tubulin of brain. The identity of the other minor polypeptides in the yeast tubulin preparation is unknown.

Effect of Benzimidazole Fungicides and Colchicine on Yeast Tubulin Assembly. Mutants of Aspergillus which are resistant to benomyl (Davidse & Flach, 1977) have been isolated. Benomyl breaks down in aqueous solution to MBC and butyl isocyanate (Hammerschlag & Sisler, 1973). These mutants have alterations in the β subunit of tubulin (Sheir-Neiss et al., 1978), suggesting that MBC inhibits mitosis by binding to β-tubulin. An MBC binding component was found in Aspergillus which might be tubulin, but it was not purified (Davidse & Flach, 1977). A variety of benzimidazoles inhibit brain tubulin assembly (Friedman & Platzer, 1978; Ireland et al., 1979), but MBC, economically the most important of them (Bent, 1979), is not an effective inhibitor. It was assumed that this was due to evolutionary differences between brain and fungal tubulin. That this is the case is confirmed

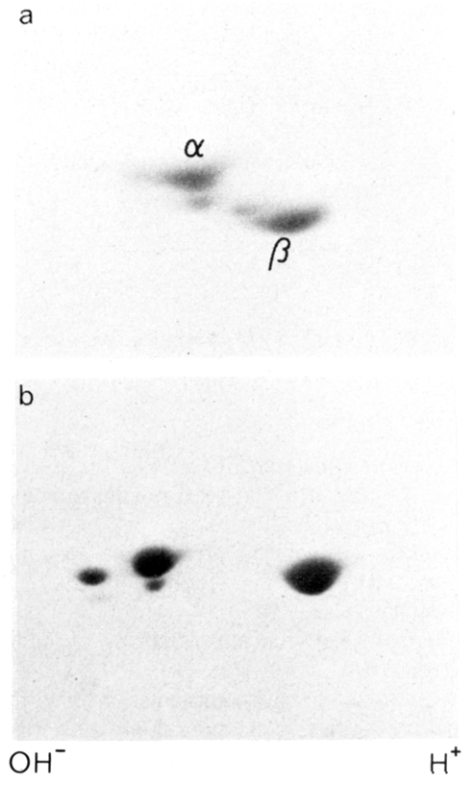

FIGURE 3: Two-dimensional gel electrophoresis of (a) brain tubulin and (b) yeast tubulin. The brain tubulin subunits are identified in (a). The same region of the gel is shown in each case.

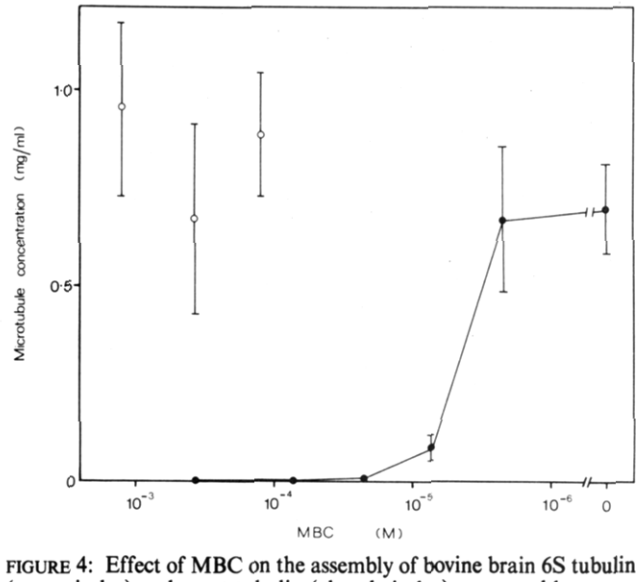

FIGURE 4: Effect of MBC on the assembly of bovine brain 6S tubulin (open circles) and yeast tubulin (closed circles) measured by quantitative electron microscopy. Protein concentration 2 mg/mL.

in Figure 4 where MBC is shown to be an effective inhibitor of yeast tubulin assembly, 50% inhibition occurring at 4×10^{-6} M MBC. MBC at 300-fold higher concentration (1.3 mM, its maximum solubility) had no effect on MAP free brain tubulin assembly (Figure 4). The brain tubulin was used MAP free to raise its critical concentration to the level for the probably MAP free yeast tubulin (about 1 mg/mL as determined by dark-field light microscopy) and also to make the two preparations more comparable in terms of associated proteins.

In Table I are shown the effects on yeast and brain tubulin assembly of another fungicidal benzimidazole, nocodazole, and also colchicine. The observed effects of the inhibitors on yeast

Table I: Effect of MBC, Nocodazole, and Colchicine on Yeast and Brain Microtubule Assembly

inhibitor	concn at which tubulin assembly is inhibited 50% (M)		concn at which yeast growth is inhibited 50% (M)
	brain	yeast	
MBC	$>1.3 \times 10^{-3}$	4×10^{-6}	3×10^{-5}
nocodazole	7×10^{-6}	1×10^{-6}	4×10^{-6}
colchicine	3×10^{-6}	2×10^{-3}	$>1 \times 10^{-3}$ [a]

[a] Haber et al. (1972).

tubulin assembly in vitro are in qualitative agreement with their in vivo effects on yeast cell division.

Discussion

Comparison of Yeast and Brain Tubulin. In this paper it is shown for the first time that yeast tubulin can be assembled in vitro into microtubules by methods used for the assembly of brain tubulin. The yeast tubulin subunits are similar to but not identical with the brain tubulin subunits on one- and two-dimensional gels.

The differences between yeast and brain tubulins can be summarized:

(i) Self-assembled yeast microtubules have mainly 12 protofilaments on the first cycle and mainly 13 protofilaments on the second cycle. Brain tubulin has 14 protofilaments under the same conditions (Burton & Himes, 1978). The number of protofilaments in yeast microtubules in vivo is not known, but some neural microtubules have 12 protofilaments (Burton et al., 1975).

(ii) Yeast microtubules from *S. uvarum* depolymerise much more slowly in the cold than brain microtubules (see Experimental Procedures). This is probably a consequence of the fact that this yeast will grow slowly at 4 °C.

(iii) Yeast and brain tubulin have different sensitivities to fungicides and drugs (Table I). Both are sensitive to nocodazole, though yeast is more sensitive. Yeast tubulin is very sensitive to MBC; brain tubulin is not. The opposite occurs with colchicine. In its low sensitivity to colchicine, yeast tubulin resembles Tetrahymena tubulin (Maekawa & Sakai, 1978), though the sensitivity of Tetrahymena tubulin to benzimidazoles was not measured.

(iv) cDNA clones to brain α- and β-tubulin do not hybridize strongly with yeast DNA (Cleveland et al., 1980), though weak hybridization to single bands in yeast DNA was detected. This suggests significant sequence differences between the yeast and brain tubulin genes.

Despite these differences the basic structure of the yeast microtubule (Figure 2) is very similar to that of the brain microtubule; both tubulins are sensitive to nocodazole. This suggests that the differences between yeast and brain tubulin are of degree rather than substance and illustrate the basic conservation of tubulin throughout the eukaryote phyla.

Comparison of the Coassembly of Yeast and Brain Tubulin with Previous Results. The results on the coassembly of yeast and brain tubulin reported here (Figure 1, lanes 8 and 9) are partially in agreement with the results of Clayton et al. (1979). They identified yeast α-tubulin on $NaDodSO_4$ gels as a broad band (M_r ~55 000) comigrating with brain α-tubulin and yeast β-tubulin as another less prominent protein (M_r ~ 52 000) migrating just ahead of brain β-tubulin. In view of the results reported here (Figure 1, lanes 8 and 9), an alternative explanation is that the broad band identified as yeast α-tubulin actually contains both the α- and β-tubulins which would have been resolved in the $NaDodSO_4$ gel system used here. The other yeast protein (M_r ~52 000) migrating ahead of brain β-tubulin could be either a proteolytic tubulin fragment or some nontubulin component which happens to bind well to brain microtubules. Clayton et al. (1979) showed that another prominent coassembling yeast protein (M_r ~49 000) migrating below brain tubulin on $NaDodSO_4$ gels and previously identified as yeast tubulin (Shriver & Byers, 1977; Baum et al., 1978) was probably not tubulin because it did not elute with brain tubulin on a phosphocellulose column.

Applicability of This Yeast Tubulin Purification Method. This method for the purification of yeast tubulin has been successfully applied to two yeast species: *S. uvarum* (NCYC 74) and *S. cerevisiae* (SC-3). This suggests that the method should be applicable to most yeast strains. Thus, if the genetic manipulation of yeast is successful in generating tubulin mutants, then the yeast tubulin purification described in this paper will be of use in characterizing their functional defects.

Acknowledgments

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Gel to Liquid-Crystalline Transition Temperatures of Water Dispersions of Two Pairs of Positional Isomers of Unsaturated Mixed-Acid Phosphatidylcholines†

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ABSTRACT: The gel to liquid-crystalline phase transition temperatures of dispersions of mixed-acid *sn*-1,2-lecithins which contain one unsaturated and one saturated fatty acid have been studied by differential scanning calorimetry. The temperature for 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (containing no reversed isomer) was −9.3 °C while that for 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (containing 8% of the reversed isomer) was −2.6 °C. The temperature for 2-oleoyl-1-stearoyl-*sn*-glycero-3-phosphocholine (containing 6% of the reversed isomer) was 6.3 °C while that for 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (containing 18% of the reversed isomer) was 8.6 °C. The differences in transition temperatures for the isomers of a pair containing the same two acids were consistent with those observed for positional isomers of saturated mixed-acid lecithins in that the isomer of the pair which had the longer fatty acid in the *sn*-1 position had the lower temperature. The phase transition temperatures of pairs of isomers containing palmitate and oleate at the *sn*-1 and -2 positions were different by at least 6.7 °C, while those containing stearate and oleate were different by at least 2.3 °C. Differences in the chain lengths of the fatty acids at the two positions of the glycerol appear to predominate over differences in the depths of the double bonds in the bilayer in determining the transition temperatures.

It has been observed that, when they are dispersed in water, pairs of positional isomers of saturated mixed-acid *sn*-1,2-phosphatidylcholines containing either myristate plus palmitate or stearate plus palmitate exhibit different gel to liquid-crystalline transition temperatures and different enthalpies of transition (Keough & Davis, 1979). These observations have recently been confirmed and extended by Chen & Sturtevant (1981). In these studies, a consistent pattern emerged in that it was always the member of an isomeric pair which contained the longer acid at the *sn*-1 position which had the lower T_c and enthalpy. In most instances, lipids of biological origin contain at least one acid with at least one double bond. Lipids which have double bonds have lower transition temperatures than lipids with equivalent saturated chains. The ultimate T_c of an unsaturated lecithin is dependent, however, not only on the presence or number of double bonds but also on their position in the chain (Barton & Gunstone, 1975).

It is of interest to know if the disruptive effect of the double bond would be great enough to supersede packing differences in pairs of positional isomers caused by alternating the position of the chain on the glycerol (Keough & Davis, 1979). Here we report upon the transition temperatures of two pairs of isomeric lecithins each containing a saturated (either palmitate or stearate) and an unsaturated (oleate) chain—unsaturated mixed-acid lecithins.

Materials and Methods

Preparation and Analysis of Lipids. Dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, OPPC,[1] and various fatty acids were purchased from Sigma Chemical Co., St. Louis, MO. Dioleoylphosphatidylcholine was either made by a method described before (Cubero Rubles & van den Berg, 1969; Keough & Davis, 1979) or was purchased from Sigma Chemical Co. Two batches of SOPC and one

† From the Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, A1B 3X9 Canada. *Received November 6, 1980.* This research was supported by the Medical Research Council of Canada (MA-5036) and the Canadian Lung Association. B.D.F. was the recipient of a Natural Sciences and Engineering Research Council of Canada Summer Studentship. The major portion of this work will form part of a Ph.D. Thesis to be submitted to Memorial University of Newfoundland by P.J.D.

[1] Abbreviations used: DSC, differential scanning calorimetry (ic); lc, liquid crystal(line); OPPC, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; OSPC, 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; POPC, 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine; SOPC, 2-oleoyl-1-stearoyl-*sn*-glycero-3-phosphocholine; T_c, gel to lc transition temperature; T_m, temperature of maximum heat flow into or out of a sample during a thermal event.

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