THE ROLE OF SUBUNIT ENTROPY IN COOPERATIVE ASSEMBLY

NUCLEATION OF MICROTUBULES AND OTHER TWO-DIMENSIONAL POLYMERS

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ABSTRACT The self-assembly and nucleation of two-dimensional polymers is described by a theory based on a model of rigid subunits and bonds and simple principles of thermodynamics. The key point in the theory is to separate as an explicit parameter the free energy, primarily attributed to the entropy of the free subunit, that is required to immobilize a subunit in the polymer. Quantitative relations for the association of a subunit forming a longitudinal bond, a lateral bond, or both together are obtained, which demonstrate the basis and magnitude of cooperativity. The same formalism leads to a quantitative estimate for the concentration of the small polymers that are important intermediates in nucleation. It is shown that, if the concentration of free subunits is below a certain "critical supersaturation," the concentration of some essential intermediates is too low to support any significant assembly and nucleation is blocked. If the subunit concentration is above the critical supersaturation, all of the small intermediates are sufficiently stable to form and grow spontaneously. The theory predicts a critical supersaturation of 3.5 to 7 (the ratio of subunit concentration to the equilibrium solubility) for parameters appropriate to assembly of the microtubule wall. Experimentally, nucleation and assembly of microtubules is obtained at somewhat lower concentrations, 1.5 to 3 times the equilibrium solubility. Special mechanisms that could stabilize small polymers and facilitate nucleation of microtubule assembly are suggested.

INTRODUCTION

Assembly of protein subunits to form a polymer must proceed through a series of bimolecular reactions. This principle, which is very important for consideration of assembly mechanisms and pathways, is simply a restatement of the fact that basic reaction mechanisms in solution are never higher than second order. The simultaneous collision of three or more subunits to form a trimer or larger polymer is far too rare an event to support any significant reaction. Thus, any oligomer must be assembled through a series of smaller intermediates. The specification of these intermediates and the bimolecular reactions that link them may be referred to as the pathway of assembly.

The bimolecular assembly steps can be of two types: (*a*) an intermediate can grow by stepwise addition of single subunits, or (*b*) a larger structure can be formed by pairwise interaction of preformed intermediates. In the latter case these smaller intermediates must have been assembled from bimolecular interactions.

The first intermediate in any pathway of assembly must be a dimer, because this is the only product that can be formed from two subunits. In principle there can be two or more types of dimer, the subunits being linked by different bonds. The dimer linked by the strongest intersubunit bond will be highly favored, but a small fraction of other forms should exist and this type of weak interaction may play an important role in assembly. The next intermediate may be a trimer, formed by addition of a subunit to an existing dimer, or may be a tetramer, formed by association of two dimers. For these and all larger oligomers there will be a number of isomeric forms with subunits linked by different patterns of bonds. The relative concentrations of these different isomeric forms will depend on the total intersubunit bond energy of each species, and all but one or two will be highly unfavorable.

In the present analysis we use simple principles of thermodynamics to estimate the stability of different small oligomers in order to evaluate which ones may be important intermediates in assembly. We are concerned here with the assembly of two-dimensional (2-D) polymers, and will use the wall of the microtubule as a specific example. In this application we will ignore most of the complexities of microtubule assembly, such as microtubule-associated proteins, ligand and drug interactions, nucleotide hydrolysis, tubulin rings, and conformational changes. Our aim is to develop the simplest model for the self-assembly of identical subunits, and to see to what extent this simple theory can explain the observed features of microtubule assembly.

A brief description of the assembly of an isotropic 2-D polymer has already been presented (Erickson, 1980) and a derivation similar to the theory presented here has been developed and applied to the nucleation of helical fibers of sickle cell hemoglobin (Eaton and Hofrichter, 1978; Ferrone et al., 1980). It will be seen that the theory is quite general and can be applied to any 2-D, tubular, or helical polymer, or to nucleation of a three-dimensional crystal, once the pattern of intersubunit bonds is specified.

THE STRUCTURE OF A 2-D POLYMER (MICROTUBULE WALL) AND ITS SMALLER INTERMEDIATES

The stability of any polymer is determined by the sum of the intersubunit bond energies, so it is necessary to specify the number and strength of the different bonds. A simple 2-D lattice of subunits, based on the structure of the microtubule wall (Erickson, 1974), is shown in Fig. 1. The fact that tubulin subunits are of two types, alpha and beta tubulin, and that assembly probably occurs by way of alpha-beta heterodimers, is ignored here. The results are virtually the same and the explanation is simpler and more general if we assume that the individual morphological subunit seen in the lattice is the chemical subunit giving rise to the assembly.

Each subunit within the wall is connected to four neighbors by two types of contacts or bonds. The most prominent is the longitudinal bond, represented by the double line, connecting subunits into protofilaments. The second type of contact is the lateral bond between subunits in adjacent protofilaments, represented here as a single line between subunits in the obliquely horizontal direction. A third type of bond, diagonal, is ignored in the present derivation because (*a*) it is not apparent in the reconstructed images, and (*b*) trial calculations have shown that the effects of adding a diagonal bond are not much different from changing the relative strengths of the two bonds. Thus the model of subunits connected

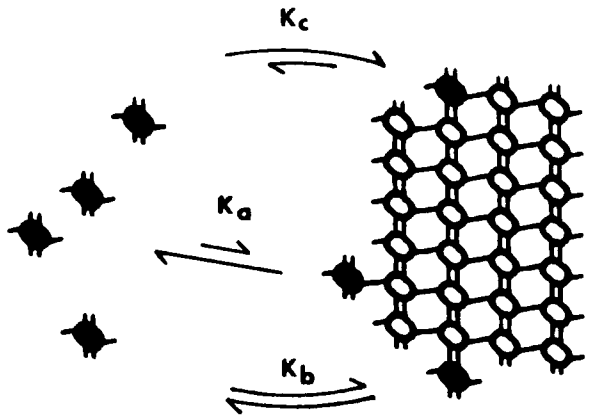

FIGURE 1 Three possibilities for assembly of a subunit into a 2-D lattice, like that of the microtubule wall. Subunits are connected to each other by two types of bonds. Longitudinal bonds connecting the subunits into vertical protofilaments are represented by a double line and lateral bonds are represented by a single line. A subunit may polymerize into this lattice forming (*a*) a lateral bond, (*b*) a longitudinal bond, or (*c*) both a lateral and a longitudinal bond. The relation of the three equilibrium constants to each other and to the bond energies is derived in the text.

by longitudinal and lateral bonds of different strengths is a good approximation for any 2-D polymer.

In the simple model of assembly developed here we assume that all intermediates are constructed from subunits joined by the same longitudinal and lateral bonds as in the final microtubule wall. These intermediates will include filaments of subunits joined by a single type of bond, and small 2-D polymers. In the case of microtubules, and indeed most other 2-D polymers, the sheet of subunits is curved and eventually closes to form an intact cylinder. The structure may then be described as a helical lattice. This closure is not, however, essential to assembly and can play little or no role in nucleation, which involves the assembly and growth of polymers too small to form the closed helical lattice (Erickson, 1978). Thus, that the microtubule is a helical polymer is largely irrelevant to its assembly, and in particular to nucleation, which is essentially the formation and growth of a 2-D polymer, the microtubule wall.

THE THERMODYNAMIC BASIS OF COOPERATIVE ASSEMBLY: RELATION OF ASSOCIATION CONSTANTS, BOND ENERGY, AND SUBUNIT ENTROPY

Cooperativity means that the association of subunits is enhanced as their number increases, i.e., that larger polymers are more favorable than smaller. The reason is that subunits in the interior, which are relatively more numerous in larger polymers, have more bonds than subunits at the edge, so the average bond energy per subunit increases as the polymer grows larger. For a quantitative description of cooperativity we need to know the free energy change for subunits associated by different numbers and combinations of bonds. The basis of the theory is illustrated most simply by considering the relation of the three association constants

for polymerization of a subunit into a 2-D lattice (Fig. 1). It can attach (*a*) to the side of the sheet, forming a lateral bond of energy e_a; (*b*) to the end of a protofilament, forming a longitudinal bond of energy e_b; or (*c*) into a niche or "cozy corner," forming both a lateral and a longitudinal bond, with total bond energy $e_c = e_a + e_b$.

The key point in the analysis is to separate the free energy of association into two parts. One component, which opposes association, is the free energy required to immobilize a subunit in the polymer. We assume that this free energy, which we designate e_s, is the same regardless of whether the subunit forms a lateral bond, a longitudinal bond, or both at once. The other component, which favors association, is the free energy associated with the interface or bond between subunits, either e_a, e_b, or $e_a + e_b$, depending on the type of bonds formed.

The most important known contribution to the e_s term is the translational and rotational entropy of the free subunit that is lost when the subunit is immobilized in the polymer. In an earlier paper (Erickson, 1980) the free energy e_s was referred to as TS_i, where S_i is the intrinsic entropy of the free subunit. The free energy corresponding to this entropy has been treated by Doty and Myers (1953) and Steinberg and Scheraga (1963). A more recent treatment by Chothia and Janin (1975), which is based on the concept of the intersubunit bond as a highly specific and rigid interface, gives a value of 20–30 kcal/mol for the free energy required to immobilize a subunit in a dimer or polymer. This value is reduced to 13–15 kcal/mol if it is assumed that the subunits in the polymer are free to undergo ~0.1 nm vibrational motions (Erickson, 1979). Even this value is quite high and we have found, in various calculations based on the theory presented here, that a much lower value of e_s, in the range of 2 to 7 kcal/mol, is needed to obtain a reasonable model for self-assembly and nucleation. In the model for nucleation of sickle cell hemoglobin assembly, very similar in principle to that presented here, Ferrone et al. (1980) treated this entropic term as an adjustable parameter. They found that a value of 6 kcal/mol ($\mu_{TR} - \mu_{PV}$, legend to their Fig. 8) gave the best fit to experimental data. For the present theory we will also treat this as an adjustable parameter and simply note that the entropic contribution may be overestimated in the theoretical derivations or may be compensated by other, unspecified factors. We will continue to refer to this as the entropic parameter, but e_s will be defined more generally as the free energy required to immobilize a subunit in a polymer. We assume that e_s is independent of the strength or number of the intersubunit bonds. This is consistent with the concept that each bond is a rigid interface, so that the subunit is immobilized to the same extent by one bond as by two or more.

The second component of the free energy of association is the bond energy, e_a, e_b, or e_c, which we define as the total free energy change involved in forming the subunit interface that constitutes each bond. This term is attributed largely to the "hydrophobic interaction" and may be roughly proportional to the surface area that is buried in the interface (Chothia and Janin, 1975; but see also discussion after Lesk and Chothia, 1980). An important assumption in the simplest development of this theory is that in the type *c* association the two bonds can both be made with no distortion, so that the total bond energy in this case is simply $e_c = e_a + e_b$.

With these assumptions and definitions the three association constants can be written in terms of the two bond energies, e_a and e_b, and the entropic parameter, e_s.

$$K_a = \exp\left[(e_a - e_s)/RT\right] \tag{1a}$$

$$K_b = \exp\left[(e_b - e_s)/RT\right] \tag{1b}$$

$$K_c = \exp\left[(e_a + e_b - e_s)/RT\right] \tag{1c}$$

$$K_b = K_a K_b \exp\left(e_s/RT\right). \tag{1d}$$

Note that both the bond energies and e_s are expressed as positive numbers. The bond energy, which favors association, appears in the exponential with a plus sign, and the entropic term, which favors dissociation, appears with a minus sign. The entropic parameter is extremely important in determining the cooperativity, because K_c is larger than the product $K_a K_b$ by the factor exp (e_s/RT). This factor is necessary to avoid subtracting the entropy loss twice when the subunit forms two bonds.

NUCLEATION OF ASSEMBLY

In smaller polymers a larger fraction of subunits will be at an edge where one or more bonding sites are free. The average bond energy per subunit will thus be less for the smaller polymers than for larger ones and they will be less favorable energetically. Nucleation, the initial phase of assembly, is the formation and growth of the unstable small intermediates, which must precede the larger, more stable polymers.

In certain conditions one can obtain a supersaturated solution of subunits considerably exceeding the concentration that would remain in equilibrium with large polymers if these existed. If a small number of seeds, short fragments obtained from a sonicated preparation of polymers, are added to this supersaturated solution, a rapid polymerization occurs onto the ends of these seeds and the subunit concentration falls to a value, C_c, which is the equilibrium subunit solubility (Oosawa and Higashi, 1967). The polymerized state is thus the state of thermodynamic equilibrium, but the system is unable to attain this state in the absence of added seeds, because the formation of the small intermediates is too unfavorable.

In other conditions, tubular and helical polymers may assemble spontaneously from a solution of subunits, without the need for added seeds. This process is called spontaneous or homogeneous nucleation, and requires that one start with a supersaturated solution of subunits, i.e., a concentration higher than the equilibrium subunit concentration, C_c. The supersaturation is required for nucleation because the small, unstable intermediates are only formed in significant number if the subunit concentration is relatively high.

The situation is similar to the nucleation of vapor condensation, which has been analyzed extensively over the past forty years (for recent work and reviews see Zettlemoyer, 1969; Reiss, 1977; Nishioka and Pound, 1977; see also Abraham, 1974 for a sophisticated treatment of the three-dimensional problem of homogenous nucleation). In a purified system, vapor can be compressed well beyond the saturation vapor pressure of the liquid phase of the same substance to obtain a supersaturated state. Condensation must begin with the formation of small drops, which are thermodynamically unstable because of the large fraction of molecules on the surface. Growth of the small drops by stepwise addition of molecules is also unfavorable until a certain size is reached. Once this size is reached, each larger drop is more stable than its smaller precursor and growth proceeds spontaneously. Any of these drops, for which

spontaneous growth is favorable, may serve as a seed or nucleus for condensation of vapor. The smallest such nucleus, which is the least stable (as defined in the next section) intermediate in the pathway, is called the "critical nucleus."[1] In general, the rate of condensation is limited by the concentration of critical nuclei that can be formed in equilibrium with the given supersaturated vapor. If this concentration is too low, spontaneous nucleation will not be obtained in a given time of observation and the supersaturated vapor will remain in metastable equilibrium. To obtain spontaneous nucleation, the vapor pressure must be above a "critical supersaturation," at which the critical nuclei can form in some significant number.

THE BASIS FOR A THERMODYNAMIC ANALYSIS OF NUCLEATION

Whether or not spontaneous nucleation occurs is essentially a question of kinetics, as any system should reach equilibrium given sufficient time. A calculation of the time-course of the reaction would require a specification of the kinetic parameters for each of the bimolecular reactions. A much simpler thermodynamic analysis can, however, provide important information on the assembly pathway. In the simplest approach it is assumed that a population of small intermediates is quickly formed in equilibrium with the supersaturated solution of subunits. The rate-limiting step in the assembly is the addition of a single subunit to the least stable intermediate, or critical nucleus. The overall rate of assembly is then proportional to $[P_n^*]\ [P_1]\ k_+$, where $[P_n^*]$ is the concentration of the critical nucleus, $[P_1]$ is the concentration of free subunits, and k_+ is the second order rate constant for addition of a subunit to the growing polymer. It will be assumed that k_+ is the same for each step.

The basis for this thermodynamic analysis is to determine the concentration of each small intermediate that could be formed in equilibrium with a given supersaturated concentration of free subunits. This value will be called a "pseudo equilibrium concentration" to emphasize that it refers to each polymer species considered individually, rather than the equilibrium of the whole system. As the reaction approaches true equilibrium, the concentration of free subunits will fall toward the equilibrium solubility, C_c, and the small polymers will disappear. The pseudo equilibrium concentration of large polymers is too high to be physically meaningful in the supersaturated solution, but will fall to reasonable values as the concentration of free subunits approaches C_c.

The pseudo equilibrium concentration is a quantitative measure of the stability of each small polymer. The value is especially important in nucleation theory, because it is the maximum concentration of an intermediate that can be formed in a given supersaturated concentration of subunits. If a particular oligomer is too unstable to exist at a "reasonable" concentration in the initial supersaturated solution of subunits, it can be excluded as an intermediate in the assembly pathway. It is, of course, necessary to specify what constitutes a "reasonable" concentration for the critical nucleus. The kinetic argument in the next section

[1]Note that the "critical nucleus" as defined here is different from the term "nucleus" as used in previous discussions of assembly of helical polymers (Oosawa and Kasai, 1962; Wegner and Engel, 1975). In these works nuclei included only stable polymers, in particular the first stable intermediate, which is one subunit larger than the critical nucleus. Our usuage of the word nucleus to include both the critical nucleus and the larger, stable nuclei is consistent with that in most treatments of homogeneous nucleation.

provides an order-of-magnitude estimate for the minimum concentration of critical nuclei needed for nucleation.

WHAT IS THE MINIMUM REASONABLE CONCENTRATION FOR THE CRITICAL NUCLEUS?

We assume that a population of small polymers, including the critical nucleus, is established quickly after the assembly reaction is initiated and that the concentration of each small polymer approaches and is maintained at the pseudo equilibrium concentration for some time, Δt, referred to as the nucleation phase. The argument is simplest for a pathway involving only sequential addition of single subunits. In this case, if we assume that the addition of a single subunit converts a critical nucleus into a stable nucleus, and that each of these stable nuclei eventually grows into a microtubule, we can calculate the minimum concentration of critical nuclei needed to produce the final population of microtubules in the specified time.

The reaction may be written

$$\text{subunits} \rightleftharpoons \underset{\text{nucleus}}{\text{critical}} \xrightarrow{k_+} \underset{\text{nucleus}}{\text{stable}} \rightarrow\rightarrow MT$$

$$P_1 \rightleftharpoons P_n^* \xrightarrow{k_+} P_{n+1} \rightarrow\rightarrow MT$$

The rate of formation of stable nuclei is then $[P_1]\,[P_n^*]\,k_+$. If the nucleation phase continues for a time Δt, the total concentration of stable nuclei or seeds, each of which will eventually produce a microtubule, will be $[P_1]\,[P_n^*]\,k_+\,\Delta t$.

In a typical microtubule assembly experiment the initiation phase lasts on the order of Δt = 100 s, and the concentration of microtubules obtained is 10^{-9} M (this corresponds to 1 mg/ml protein assembled into microtubules 5 μm long). The second-order rate constant for addition of subunits onto the ends of microtubules is $\sim 1\text{–}5 \times 10^6\ M^{-1}\ s^{-1}$ (Binder et al., 1975; Bryan, 1976; Johnson and Borisy, 1977). This is similar to the fastest rates observed for several other protein-protein interactions and is probably close to the maximum, diffusion-limited reaction rate (Koren and Hammes, 1976). A value of $k_+ = 5 \times 10^6\ M^{-1}\ s^{-1}$ is a reasonable estimate for the maximum rate of conversion of critical nuclei into seeds. Setting $[P_1] = 10^{-5}$ M and Δt = 100 s, we see that to produce 10^{-9} M microtubules requires a concentration of critical nuclei such that $10^{-9} = [P_n^*]\,(10^{-5})\,(5 \times 10^6)\,(100)$; $[P_n^*] = 2 \times 10^{-13}$ M.

This is the order-of-magnitude estimate for the minimum stationary concentration of the critical nucleus necessary to produce the 10^{-9} M stable nuclei of microtubules in the 100 s of the nucleation phase. Any oligomer whose pseudo equilibrium concentration is much less than this value may be excluded as a possible intermediate in the pathway of assembly, because it cannot exist in sufficient numbers to serve as a source of seeds and microtubles.

THE PSEUDO EQUILIBRIUM CONCENTRATION OF SMALL, TWO-DIMENSIONAL POLYMERS

A quantitative measure of the stability of a particular polymer, which we will use in the following discussion, is the concentration that could be assembled in equilibrium with a given supersaturated concentration of free subunits, defined above as the pseudo equilibrium

concentration. As this is a thermodynamic or equilibrium measure, we can ignore the actual pathway of bimolecular reactions and write the assembly as a single reaction.

$$ijP_1 \overset{K(i,j)}{\rightleftharpoons} S(i,j) \tag{2a}$$

$$[S(i,j)] = [P_1]^{ij} K(i,j). \tag{2b}$$

$[P_1]$ is the concentration of free subunits, $[S(i,j)]$ is the concentration of rectangular sheets i subunits wide by j subunits long, and $K(i,j)$ is an equilibrium constant characteristic of each polymer. (A statistical factor $2^{(ij-1)}$ should be added to Eq. 2b if subunits can assemble onto the sheet in a nonpolar manner. This will change the relative concentrations of the different polymers by a factor on the order of two to ten, which can be neglected for the order of magnitude estimates of the present discussion.)

$K(i,j)$ may be calculated by the same formalism used above to determine the three association constants. The total negative free energy for the assembly of the ij subunits into the sheet is simply the sum of all bond energies minus the entropic energy for each subunit immobilized in the polymer.

$$K(i,j) = \exp\{[\Sigma e - (ij - 1)\, e_s]/RT\} \tag{2c}$$

$$K(i,j) = \exp\{[ij(e_a + e_b - e_s) - ie_b - je_a + e_s]/RT\}. \tag{2d}$$

The entropic parameter e_s has been subtracted once for each subunit in the polymer and added back once to account for the entropy of the polymer itself. (The entropy of the polymer is somewhat greater than that of a single subunit, but this difference is not significant for the present calculation.) Eq. 2c is quite general and may be applied to any polymer if the number and strength of the intersubunit bonds can be specified.

NUMERICAL CALCULATIONS

To calculate the association constants $K(i,j)$ we need to specify the parameters e_s, e_a, and e_b. As discussed above, a value for the entropic parameter e_s in the range of 2 to 7 kcal/mol will give a reasonable model for nucleation and assembly. We will present calculations for two values, 2.1 and 6.9 kcal/mol, to show the effect of this parameter in the theory. The bond energies e_a and e_b can be estimated from two observable parameters: the ratio of the bond energies is indicated by the ratio of length to width of the 2-D polymers, and the sum of the bond energies may be determined from the concentration of subunits left in equilibrium with very large polymers, C_c. Details are given in the next two paragraphs.

The longitudinal bond appears to be considerably stronger than the lateral bond because images of disintegrating microtubules commonly show the wall fraying into protofilaments, which remain intact and apparently stable. More quantitatively, one can derive from Eq. 2c that the most stable sheet formed from a given number of subunits will have a length to width ratio equal to the ratio of bond energies, $j/i = e_b/e_a$ (Carlier and Pantaloni, 1978). In negatively stained specimens, sheets are always observed to be considerably longer than they are wide. The exact ratio probably varies with assembly conditions, but it is on the order of 5–10. We will use the value $e_b/e_a = 5$ in the calculations here.

At the end of the assembly reaction there will be a pool of subunits, at concentration $[P_1] =$

C_c, in equilibrium with very large polymers. For very large ij, Eqs. 2b and d give

$$[S(i,j)] = \{[P_1] \exp [(e_a + e_b - e_s)/RT]\}^{ij} = \{[P_1]\, K_c\}^{ij}. \quad (3)$$

Because of the exponential behavior the equilibrium concentration of sheets is extremely large or small unless $[P_1]\, K_c$ is very close to unity, so the equilibrium concentration of subunits must be

$$C_c = K_c^{-1} = \exp [-(1/RT)\,(e_a + e_b - e_s)]. \quad (4)$$

This equilibrium or critical concentration for assembly of tubulin depends on solution conditions (Lee and Timasheff, 1975; Himes et al., 1977; Herzog and Weber, 1977), but is typically on the order of 10^{-5} M. Setting $C_c = 10^{-5}$ M, $e_b = 5e_a$, and $e_s = 2.1$ or 6.9 kcal/mol, we determine the bond energies e_a and e_b using Eq. 4. Numerical values for e_a and e_b, as well as the association constants K_a, K_b, and K_c, are tabulated in Table I.

THE PSEUDO EQUILIBRIUM CONCENTRATION OF SMOOTH-SIDED SHEETS

The concentrations of small filaments and sheets that can be formed in equilibrium with a supersaturated pool of subunits were calculated from Eqs. 2b and d; values are shown graphically in Fig. 2. The relative concentration of free subunits is expressed as $[P_1]/C_c$, the supersaturation ratio, and is given on a logarithmic scale on the abscissa. Two graphs are given, one for the case $e_s = 2.1$ kcal/mol, the other for $e_s = 6.9$ kcal/mol. We will discuss the first case, Fig. 2 *a*, in detail.

The most important feature of the graph is the point at $[P_1]/C_c = \exp(e_a/2RT) = 3.5$, where two families of lines all intersect. At this concentration of free subunits, all sheets with two protofilaments and all sheets in which the protofilaments are 10 subunits long exist at the same concentration: $[S(2,j)] = [S(i,10)] = 5 \times 10^{-10}$ M. We identify this value of $[P_1]$ as the "critical supersaturation," because for a subunit concentration below this value the smallest 2-D polymers, in particular the two-filament sheets, will tend to disassemble rather than grow spontaneously. The pseudo equilibrium concentration decreases as the polymer grows larger, and it may be seen in Fig. 2 *a* that for many of the intermediate sized polymers, such as $S(2,5)$, $S(2,10)$, and $S(3,10)$, the concentration is below the 2×10^{-13} M needed to support nucleation. On the other hand, if $[P_1]$ is greater than the critical supersaturation, the pseudo

TABLE I
BOND ENERGIES AND ASSOCIATION CONSTANTS FOR MICROTUBULE ASSEMBLY

e_s(kcal/mol)	6.9	2.1
e_a(kcal/mol)	2.3	1.5
e_b(kcal/mol)	11.5	7.5
K_a(M^{-1})	4.7×10^{-4}	0.37
K_b(M^{-1})	2.1×10^3	8.1×10^3
K_c(M^{-1})	10^5	10^5

Bond energies and association constants calculated from Eq. 4 for the case $e_b = 5e_a$, $C_c = K_c^{-1} = 10^{-5}$ M, and for e_s equal to 6.9 or 2.1 kcal/mol.

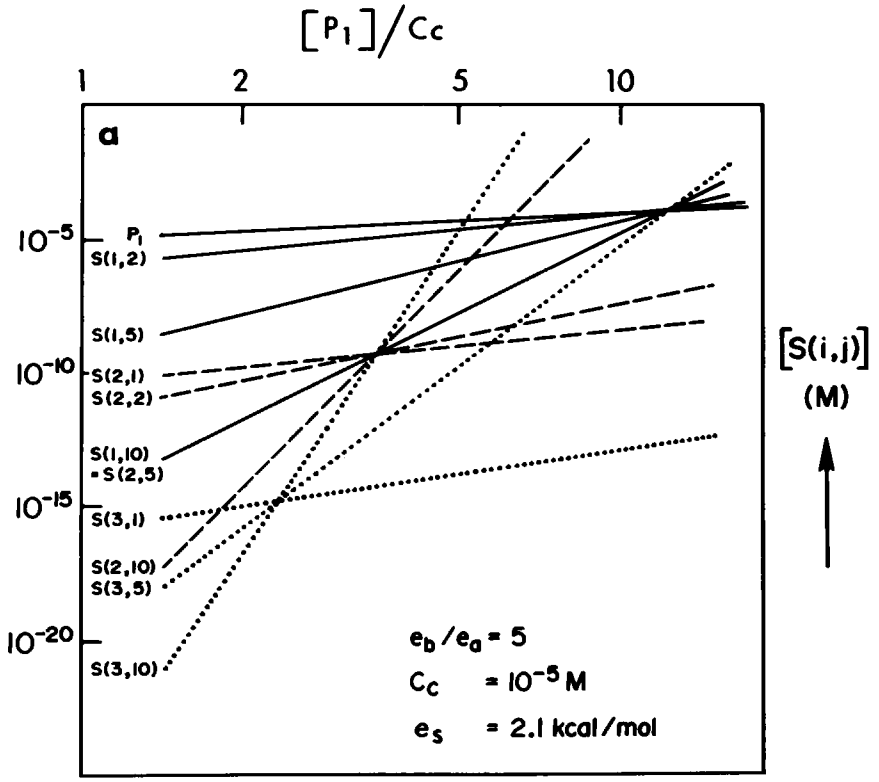

FIGURE 2*a*

FIGURE 2 Numerical values for the pseudo equilibrium concentration of small one- and two-dimensional polymers as a function of subunit concentration. The concentration of free subunits is expressed on the abscissa on a logarithmic scale as the ratio of supersaturation, $[P_1]/C_c$. The concentration of each polymer is given on the ordinate on a much steeper logarithmic scale. The values were calculated from Eq. 2 for the case $e_b/e_a = 5$, $C_c = K_c^{-1} = 10^{-5}$ M, and either $e_s = 2.1$ kcal/mol (Fig. 2 *a*), or $e_s = 6.9$ kcal/mol (Fig. 2 *b*).

equilibrium concentration increases progressively as the two-filament sheet elongates, i.e., $[S(2,j + 1)] > [S(2,j)]$. Thus elongation of this 2-D polymer will be more favorable than shortening and may occur spontaneously by addition of subunits, as described in the next section. Similarly, for sheets longer than 10 subunits, lateral growth will be favored, although the pathway may be more complicated. In summary, for a subunit concentration below the crossover point that we have identified as the critical supersaturation, the smallest 2-D polymers will tend to disassemble and many important intermediates can not be formed in sufficient number to support nucleation, whereas for higher subunit concentration, they will be stable and tend to grow spontaneously.

For the larger value of the entropic parameter, $e_s = 6.9$ kcal/mol, the critical supersaturation is $[P_1]/C_c = 6.8$, and the concentration of small polymers at this crossover point is only $\sim 10^{-12}$ M, near the minimum reasonable concentration (2×10^{-13} M) for an intermediate. For values of $e_s > 7$ kcal/mol, the theory developed here would not predict spontaneous nucleation except for extremely high concentrations of free subunits.

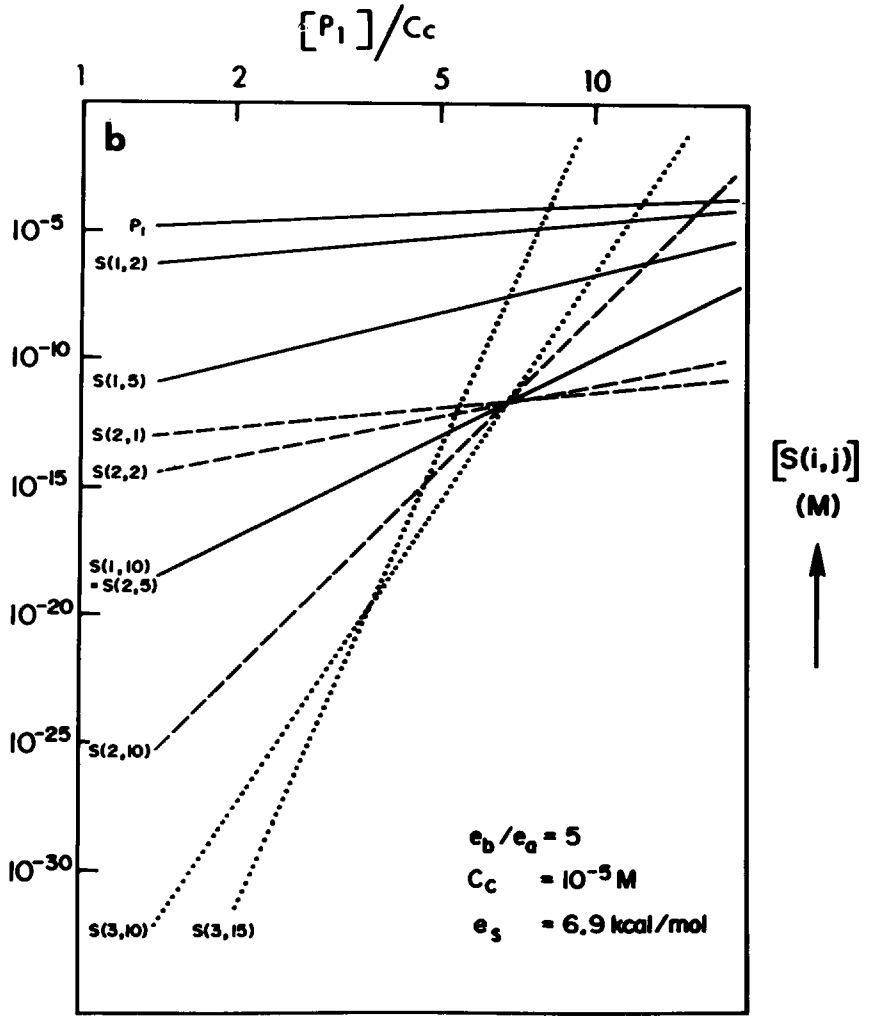

FIGURE 2*b*

DETAILED PATHWAYS FOR LONGITUDINAL AND LATERAL GROWTH

The sheets $S(i,j)$ described by Eq. 2 and discussed in the previous section are all smooth-sided, so longitudinal or lateral growth to the next larger form requires addition of a complete row or column of subunits. As discussed in the Introduction, the actual growth must proceed through a series of bimolecular reactions, either the sequential addition of single subunits or the attachment of a preformed filament in a single step. The detailed pathway for longitudinal and lateral growth of the 2-D polymer probably involves both of these alternatives, as described below.

Longitudinal growth of a single filament and two-filament sheets by sequential addition of subunits is illustrated in Figs. 3 *a* and *b*. Fig. 3 *b–d* shows three possible pathways for lateral growth, in particular for the first step, the formation of a short, two-filament sheet. The

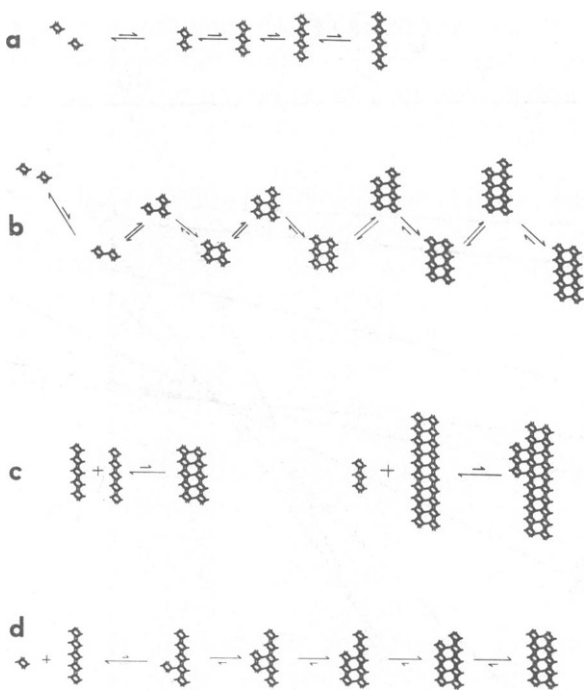

FIGURE 3 Proposed pathways for longitudinal and lateral growth in nucleation of a 2-D polymer. The relative length of the arrows for the forward and reverse reactions indicates the favored direction of each step, at a subunit concentration near the critical supersaturation.

stability of the different intermediates involved in these pathways has been evaluated by calculating the pseudo equilibrium concentration as in the previous sections. Instead of presenting a detailed derivation of these numerical results, which must be based on speculative choices for certain parameters, we prefer to describe the main features of the pathways qualitatively and to present only the important conclusions from the quantitative analysis.

Short longitudinal filaments are the most favorable species in terms of concentration. A population of these filaments should be established quickly after buffer conditions are changed to favor assembly. Longitudinal growth of single filaments (Fig. 3 *a*) is unfavorable, in the sense that the pseudo equilibrium concentration decreases as the length of the filament increases. Filaments up to 10 subunits long can, nevertheless, exist at concentrations $>10^{-10}$ M, in equilibrium with the pool of subunits. These filaments serve as essential intermediates for lateral growth in pathways *c* and *d*.

The pseudo equilibrium concentration of all two-filament sheets is around 5×10^{-10} for a subunit concentration near the critical supersaturation. This is much less than the concentration of short single filaments, but in contrast to the single filaments, the concentration of two-filament sheets increases as the sheets grow longer. Elongation of these sheets should therefore occur spontaneously by sequential addition of subunits (Fig. 3 *b*) so long as the concentration of free subunits is maintained above the critical supersaturation. Assembly of the two-filament sheet may actually be initiated by the lateral dimer, which can also exist at 5×10^{-10} M. Longitudinal growth of wider sheets will occur by the same pathway, sequential

addition of subunits. Sheets wider than three filaments cannot, however, be initiated by this mechanism, because the lateral trimers or larger lateral filaments are so unfavorable that their concentration is well below 10^{-13} M. In general, wider sheets must come from lateral growth, starting with single filaments or two-filament sheets, by the pathways illustrated in Fig. 3 *c* and *d*.

In the first of these pathways lateral growth is achieved by the association of preformed filaments (Fig. 3 *c*). The length of the filaments participating in this association is important in two ways. The lateral association becomes thermodynamically more favorable as the filaments become longer, because there are more lateral bonds formed in the single association step. The kinetics of their association is decreased, however, because the concentration of the filaments is greatly reduced as their length increases. Eventually the rate of association falls too low to make a significant contribution. For the free energy parameters used here the association of two five-subunit filaments or the initiation of a new protofilament by attachment of a three-subunit filament, also illustrated in Fig. 3 *c*, are reasonable both thermodynamically and kinetically. In general, the contribution of longer or shorter filaments will depend on the relative kinetics of association of filaments of different length. Values for these kinetic parameters are entirely speculative at present.

In pathway *d* (Fig. 3) the lateral growth is initiated by the attachment of a single subunit to the side of a filament, or to a smooth-sided sheet, and the new protofilament is then built by stepwise addition of subunits to this critical nucleus. The pseudo equilibrium concentration of this critical nucleus will be equal to $j\ K_a\ [P_1]\ [S(i,j)]$, where $S(i,j)$ is the smooth-sided sheet from which it is formed. The factor j is included because there are j possible sites for the attachment of the subunit. For sheets 10 subunits long ($j = 10$) and values taken from Fig. 2 *a*, this concentration will be (10) (0.37) (3.5×10^{-5}) (5×10^{-10}) = 6.5×10^{-14}. This is slightly less than the 2×10^{-13} M estimated earlier as the minimum reasonable concentration for a critical nucleus, from which we conclude that this pathway is at the limit of feasibility as a general mechanism for lateral growth. Unless the kinetics of association of preformed filaments are very much slower than the association of single subunits, pathway *c* should be much more important for lateral growth, and pathway *d* may make a negligible contribution.

THE PATHWAY OF ASSEMBLY AND NUCLEATION

In summary, we propose the following as a general pathway for nucleation of the 2-D polymer. Starting with a solution of subunits well above the critical supersaturation, assembly is initiated by suddenly changing the buffer conditions (e.g., raising the temperature). A population of longitudinal filaments, one to ten subunits long, is built up quickly after assembly is initiated. The pool of free subunits is depleted somewhat but remains above the critical supersaturation. A crucial step is the formation of the first 2-D polymer, a two-filament sheet. Under some circumstances this may occur through pathway *b* (Fig. 3), which involves only the stepwise addition of single subunits, not the pool of filaments. In general, however, the formation of a two-filament sheet of reasonable length, say five to ten subunits long, may proceed faster through pathways *c* or *d*, in which the preformed longitudinal filaments serve as intermediates.

So long as the concentration of free subunits remains above the critical supersaturation, elongation of the two-filament sheets will be more favorable than shortening and these sheets

will grow longer spontaneously. Further lateral growth requires the initiation of a third protofilament by lateral attachment of a short filament or of a single subunit (pathways *c* and *d*). This becomes more favorable as the sheet elongates, simply because there are more sites available for the initial lateral attachment.

Eventually a third protofilament will be initiated and completed, and then the stability of the sheet and the tendency toward spontaneous elongation are greatly enhanced. Specifically, for three-filament sheets, elongation will be favored over shortening for concentrations of free subunits down to $[P_1]/C_c = 2.1$ (the crossover point for $S(3, j)$, Fig. 2 *a*). This is important because the pool of free subunits will necessarily fall as the polymers are formed. As soon as it falls below the critical supersaturation, $[P_1]/C_c = 3.5$, two-filament sheets will start to shorten, but the three-filament sheets that have been formed by that time will remain stable and continue to grow. When the subunit concentration falls below $[P_1]/C_c = 2.1$, only sheets with four or more protofilaments will be stable. Eventually, as $[P_1]$ approaches C_c, all of the smaller polymers will disappear and there will be only large sheets and microtubules in equilibrium with free subunits.

DISCUSSION: COMPARISON OF THEORY WITH EXPERIMENT AND SUGGESTED MECHANISMS TO FACILITATE NUCLEATION

The theory explains the basis for cooperativity, that small polymers are thermodynamically unfavorable relative to larger ones, in a way that leads to quantitative estimates for the concentration of each species. The earlier theories of Oosawa and Kasai (1962) and Oosawa and Higashi (1967) did not treat the subunit entropy as an explicit term and therefore did not provide a natural relation among the association constants, which is needed for quantitative analysis of nucleation. The theory developed here shows that small polymers are extremely unstable relative to large ones, but it also demonstrates quantitatively that if the concentration of subunits is high enough the small intermediates should be formed in sufficient quantity to support nucleation.

One of the most important conclusions from the theory is that there is a critical supersaturation, below which spontaneous nucleation cannot be obtained. For the case of an isotropic 2-D polymer ($e_a = e_b$) the critical supersaturation was found to be 10 or 70 for e_s = 2.1 or 6.9 kcal/mol (Erickson, 1980). For an elongated polymer like the microtubule wall, with $e_b = 5e_a$, the critical supersaturation is 3.5 or 7 for the two values of e_s. Experimentally, studies of self-assembly of a variety of 2-D and tubular polymers have been conducted at relatively high protein concentrations, 10 to 100 times C_c. This is consistent with the range of critical supersaturations predicted from the theory, but in most cases the data are insufficient to specify a critical supersaturation. One of the better characterized systems is microtubule assembly, and in this case nucleation can be obtained at subunit concentrations significantly below the critical supersaturation predicted by the theory. Assembly of purified tubulin is routinely observed at concentrations in the range of 1.5 to 3 times C_c for a variety of different buffer conditions (Lee and Timasheff, 1975; Himes et al., 1977; Carlier and Pantaloni, 1978). This suggests that there is a special mechanism that facilitates nucleation of microtubule assembly at low protein concentrations. Several possible mechanisms are postulated below.

Nucleation would be facilitated if the small intermediates were able to adopt a conforma-

tion in which the intersubunit bonds were enhanced relative to their value in the large polymers. One possibility is that subunits at an edge, which are relatively more numerous in small polymers, might be able to distort their orientation to achieve a more favorable bonding interface than is possible for subunits in the middle. Another type of lattice distortion of particular relevance to microtubules is the transition of the protofilaments from the curved conformation of the rings to the straight conformation in large sheets and microtubules. Small sheets might be stabilized by adopting a spiral form that preserves the curved conformation of the protofilament (Erickson, 1978).

An assembly mechanism in which polymerization induces the subunit to change conformation to a state favoring further association has been termed "autostery" by Caspar (1980). He argued that a high degree of cooperativity (the relative instability of small polymers, which makes nucleation unfavorable) could be attributed entirely to autostery. Erickson (discussion after Caspar, 1980) noted that cooperativity could also be attributed to the entropic parameter, as elaborated here. It is interesting that actin assembly, which was the focus for that discussion, exhibits an extremely high cooperativity, and that a very large entropic parameter, e_s = 14 kcal/mol, is required to fit the data of Wegner and Engel (1975). If the entropic factor is in the range of 2 to 7 kcal/mol, as proposed here, some other mechanism, such as autostery, must play a role in generating the observed high cooperativity. It can be shown, however, that a reverse mechanism ("antiautostery") is not valid thermodynamically (Erickson, unpublished observations), so this type of mechanism cannot explain the low cooperativity, or facilitated nucleation observed with microtubules.

Another possible mechanism for facilitated nucleation of microtubule assembly is that the energy from the irreversible hydrolysis of GTP, which accompanies each polymerization step, might somehow be used to stabilize small polymers relative to large ones. The experiments of Carlier and Pantaloni (1978) on the assembly of tubulin in GDP and GTP show that nucleotide hydrolysis does play a very important role in nucleation. Tubulin-GDP is only slightly less efficient than tubulin-GTP in polymerizing onto existing microtubule seeds, but tubulin-GDP is apparently incapable of giving spontaneous nucleation at the concentrations examined so far, up to several times C_c. We can speculate that polymerization of tubulin-GDP is a truly reversible assembly and therefore requires a high supersaturation for nucleation, as predicted by the theory. An important test will be to look for spontaneous nucleation of tubulin-GDP at very high protein concentration.

Finally, a mechanism of heterogeneous nucleation, as proposed recently by Ferrone et al. (1980) to explain the kinetics of sickle cell hemoglobin assembly, may be considered. This mechanism postulates that the formation of nuclei is greatly enhanced at the surface of an existing polymer. Then, as soon as one or a few small polymers had assembled by homogeneous nucleation, these would stimulate the formation of more nuclei along their sides (heterogeneous nucleation) and the assembly reaction would take off autocatalytically. In their elegant experimental study, Ferrone et al. (1980) provided compelling evidence that the mixture of homogeneous and heterogeneous nucleation exists and is consistent with the observed kinetics of assembly.

In summary, we feel that the theory presented here, which is based on the simplest assumption of rigid subunits and bonds, is basically correct. It predicts spontaneous nucleation in the time span of a few minutes and at subunit concentrations close to those used for many

different in vitro assembly systems. That microtubule assembly is actually obtained at concentrations somewhat below the critical supersaturation predicted here is not considered a contradiction of the theory, but suggests that some additional mechanism is acting to stabilize the small polymers and enhance nucleation. The simple model of rigid subunits and bonds, and the formalism of separating the entropic parameter as an explicit term with a fixed value, provide a basis for understanding nucleation of microtubules and should be generally useful in the analysis of other cooperative self-assembly systems.

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