Topological Mechanisms Involved in the Formation of Clathrin-coated Vesicles

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ABSTRACT By examining the basic characteristics of clathrin lattices, we discover that simple topological rules impose strict constraints on clathrin lattice transformations. These constraints require that internal bond rearrangements take place in conjunction with the addition or removal of pairs of clathrin triskelions within the interior of existing clathrin lattice patches. Similar constraints also are relevant to coated-vesicle shape changes and their budding-off from pit lattices. Via specific illustrations, successive vesicles with hexagonal-barrel and other coats are shown to grow out from the interior of a initially flat clathrin-coated pit so long as free triskelions are available from cytoplasm. Concomitantly, we present mathematical derivations of several simple and useful topological equations. These equations govern the numbers of nonhexagonal clathrin lattice facets and their variations during internal shape transformations and justify the proposed mechanisms of triskelion pair insertion and removal.

I. INTRODUCTION

One of the most important mechanisms by which cells take up materials from extracellular fluid is *receptor-mediated endocytosis* involving a dynamic cycle of clathrin triskelion assembly and disassembly (Alberts et al., 1989; Pastan and Willingham, 1985; Brodsky, 1988; Pley and Parham, 1993). Clathrin triskelions first assemble on the cytoplasmic side of the cell membrane in the form of *coated pits* containing patches of more-or-less flat network, within which specific receptor molecules and their associated ligands then cluster. The coated pits subsequently become deeply invaginated and pinch off to form intracellular *coated vesicles*, carrying the accumulated receptor-ligand complexes into the cell. Finally, the coated vesicles rapidly reorganize; the ingested molecules eventually end up in designated regions of the cell, while the disassembled clathrin triskelions, the receptors, the membrane patches, etc., are returned to the plasma membrane for the next cycle of endocytosis (Alberts et al., 1989; Pastan and Willingham, 1985; Brodsky, 1988). Under normal physiological conditions as many as 1000 coated pits are present on the surface of a cell at any moment, covering approximately 1% of the total cell surface (Pastan and Willingham, 1985). Each endocytotic cycle takes about 20 s, mostly for the formation of a coated vesicle from a coated pit (Pastan and Willingham, 1985).

During the past decade this important process has been extensively studied via a range of biochemical and morphological methods. Although the broad outline of the endocytotic process as discussed above seems to be clear, many steps yet need to be clarified in detail (Schmid, 1992). One fundamental question which, until now, has remained unanswered is how essentially flat lattices of triskelions in coated pits, having hexagonal packing arrangements, transform into closed-vesicle lattices that have distinctly different topology (Heuser, 1989). In this paper we concentrate on the mechanisms of clathrin lattice transformation (i.e., shape and/or topology change) that resolve this question and lead to improved understanding of other aspects of endocytosis.

A. Clathrin triskelions and lattices

The essential units of clathrin lattices, the *triskelions*, are protein assemblies having distinct and remarkable features. Electron micrographs (Crowther and Pearse, 1981; Pearse and Bretscher, 1981; Pearse and Crowther, 1987; Kocsis et al., 1991) of individual triskelions readily reveal a characteristic pinwheel skeleton structure with three extended arms (see Fig. 1 *a*). Each arm of a native triskelion consists of one heavy chain and one tightly associated light chain (Pastan and Willingham, 1985; Brodsky, 1988; Brodsky et al., 1991). While all heavy chains (molecular mass about 190 kDa) appear to be identical, at least two classes of light chains (23–27 kDa) exist and control most essential properties of clathrin triskelions (Brodsky et al., 1991). Although considerable variation in the shape of the arms has been observed (Kocsis et al., 1991), triskelions may still be modeled as piece-wise uniform pinwheel with flexible joints (see Fig. 1 *b*) (Brodsky, 1988; Yoshmura et al., 1991). On average, each arm has a diameter of approximately 30 Å and consists of the so-called proximal section (about 170 Å in length), the distal region (about 220 Å in length), and the globular terminal domain (see Fig. 1). The proximal region, which is connected to the center of a triskelion, consists of a part of the heavy chain intertwined with a light chain, but both the distal region and the terminal domain are entirely constituted of the heavy chain (Brodsky, 1988; Pley and Parham, 1993).

The above morphology is, of course, determined by various triskelion intramolecular energetics and intermolecular interactions which still are not known precisely (Kocsis et al., 1991). For instance, the well-noted fact that triskelion pinwheels almost always exhibit the same handedness when laid down on electron microscope grids (see Fig. 1 *a*) has been explained by a standard hypothesis that the three terminal

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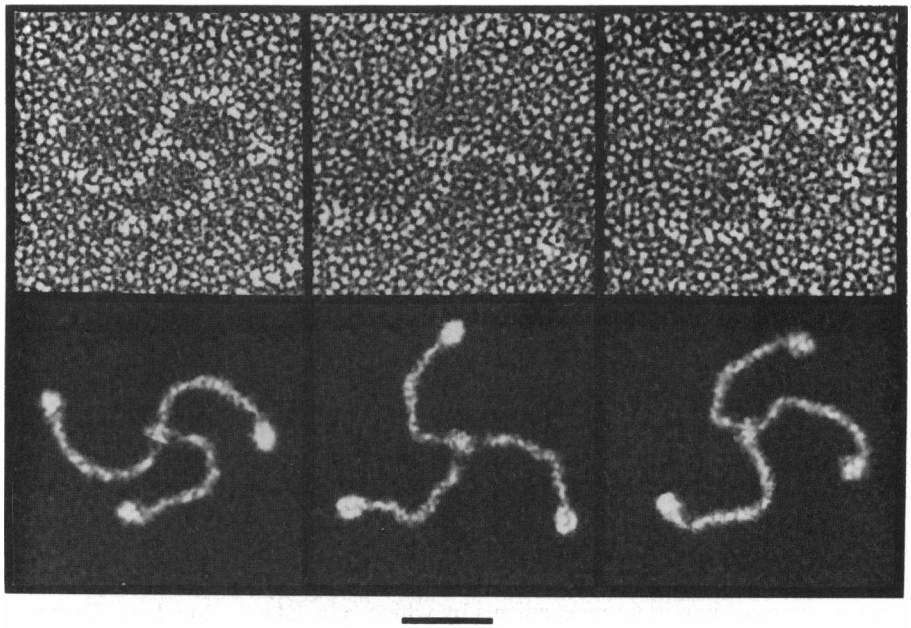
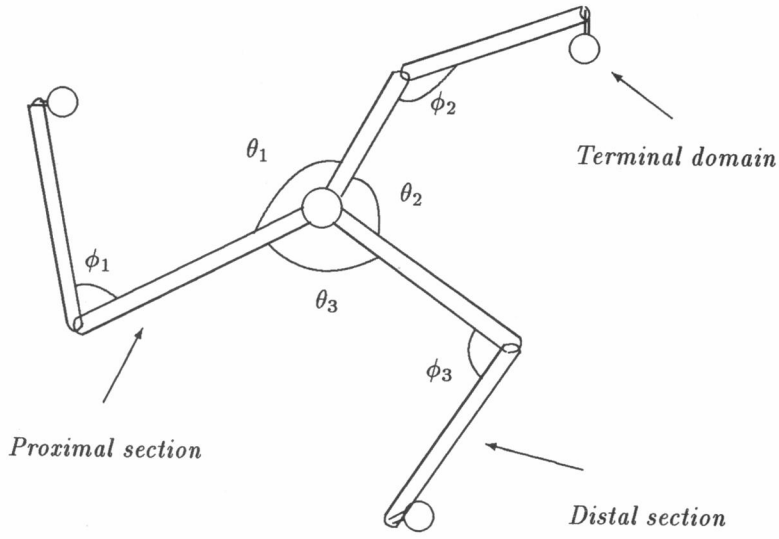


(a)



(b)

FIGURE 1 Structure of individual triskelion molecules: (*a*) electron micrographs (*top row*) of individual triskelions laid down on a mica substrate and their image-enhanced representations (*bottom row*) (courtesy of E. Kocsis), where the bar measures 250 Å; (*b*) a traditional, idealized model of a clathrin triskelion, showing functional components. In this idealized model, a triskelion has six degrees of angular freedom associated with elastic energy, with each arm section taken to be completely rigid, and the terminal domains freely rotatable. The three arms need not be in a same plane, so the relationship $\theta_1 + \theta_2 + \theta_3 = 360°$ cannot be assumed. Recent work by Kocsis et al. (1991) has shown that the three proximal sections and three distal sections have variable apparent lengths, suggesting that both proximal and distal sections are subject to bending and length variations which are greater than previously believed.

domains bend in a same direction and, then, mediate a one-sided, asymmetrical affiliation to substrates and/or cell membranes (Pearse and Crowther, 1987; Pastan and Willingham, 1985; Brodsky, 1988). More generally, this anisotropic binding and other triskelion properties reflect both energetic and entropic factors. The intramolecular energetics clearly

should involve the various angles between subregions of a three-dimensionally extended triskelion (see Fig. 1 *b*) (Yoshmura et al., 1991; Kocsis et al., 1991). The intermolecular interaction must entail an attractive component, which probably arises through the overlapping of triskelion arms.

Directed by such energetics and interactions, triskelions assemble along the cell membrane into *polygonal lattices* having varying curvatures in coated pits and coated vesicles (Kanaseki and Kadota, 1969; Heuser, 1980) (see Fig. 2 *a*). While hexagonal lattice facets most commonly are observed in electron micrographs of relatively flat coated pits, pentagonal and heptagonal facets also are frequently seen. Regardless of this variation, each vertex in the interior of the clathrin lattice always is found to connect with three polygonal edges, and each edge always measures about 170 Å in

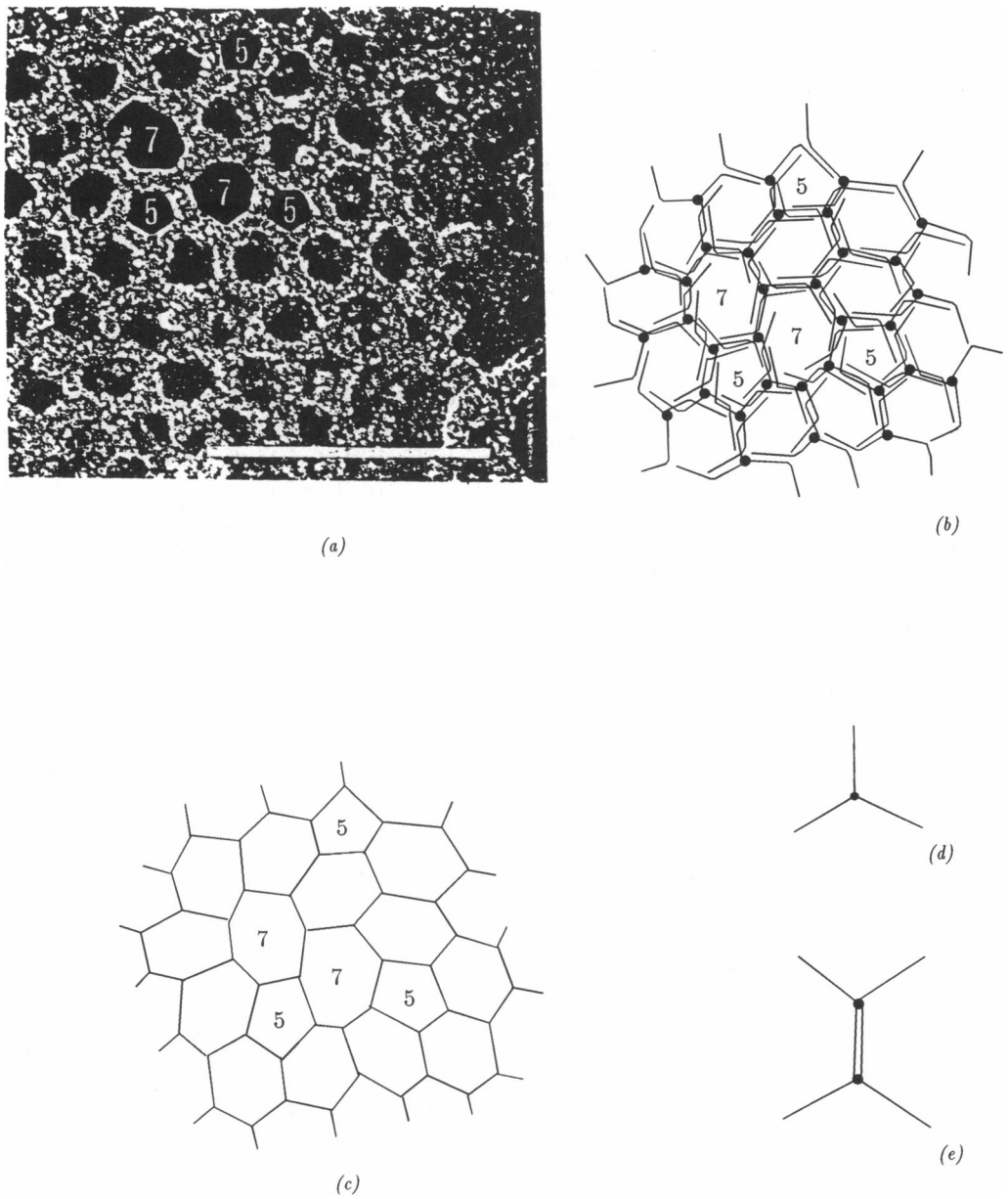


FIGURE 2 Diagrams of a clathrin lattice and the underlying packing arrangement of triskelions. (*a*) An electron micrograph of the clathrin lattice of a coated pit (from Heuser, 1980), where the white bar measures 1000 Å. While most polygonal facets are hexagons, one readily observes pentagons (numbered *5*) and heptagons (numbered *7*) in partially invaginated coated pits. (*b*) A detailed drawing showing the most probable packing structure of the triskelions in the coated pit shown in *a* (Pastan and Willingham, 1985; Pearse and Crowther, 1987). The center (*filled circles*) of each triskelion molecule, together with three terminal domains (which are bent beneath the lattice and, thus, have not been drawn explicitly) of neighboring triskelions, occupies one lattice vertex; each lattice bond (i.e., connection between lattice vertices) in the interior consists of two proximal sections and two distal sections attracted together through hundreds of atomic bonds. The polygonal lattice shown in *c* is a skeleton representation of the clathrin lattice in *a*, where triskelion molecules are reduced to *monomers* with three "sticks" (see *d*). By overlapping these sticks, each triskelion monomer at the interior of a lattice forms connections (i.e., lattice "bonds") with exactly three nearest neighbors. Similar skeleton representations will be used consistently in later figures; double lines will be used to highlight lattice bonds between pairs of molecules when they are regarded as triskelion *dimers* (see *e* and, e.g., Fig. 4).

length (Crowther and Pearse, 1981; Pearse and Bretscher, 1981; Pearse and Crowther, 1987). These observations correspond well with the general conclusion that one triskelion occupies each of the lattice vertices, that each of the triskelions arms runs along two adjacent polygonal edges with its terminal domain bending toward the underlying membrane, and that every edge in a full lattice is formed by two proximal arm sections running in opposite directions and by two distal leg sections also running in opposite directions (see Fig. 2) (Pastan and Willingham, 1985; Pearse and Crowther, 1987).

B. Clathrin lattice transformations during endocytosis

Despite the progress that has been made in establishing the structure of the pits and networks, the mechanism driving the cycle of assembly and disassembly of clathrin lattice during endocytosis is still much debated (Schmid, 1992). Various competing schemes for coated-vesicle formation have been suggested, but all entail major difficulties, as we explain below.

One proposal (McKinley, 1983) involves an energy-driven treadmilling model in which the cycling of triskelions is considered to be similar to the circulation of subunits in asymmetric head-to-tail linear polymers such as tubulin and actin (Wegner, 1976). In this model it is postulated that free triskelions are incorporated into clathrin lattices under the influence of membrane receptors. Lattice transformations, leading to increased lattice curvature and closed vesicles, are presumed to result from the rapid formation and dissolution of clathrin bonds between triskelions located at the *edge* of the lattices, but not at points *within* the lattice interior. Also, among competing models is a proposal that pentagonal lattice facets somehow diffuse from the edge of the lattice to the interior as the invagination progresses (Pearse and Bretscher, 1981; Pearse and Crowther, 1987). Note that the accumulation of pentagons is necessary for coated vesicle formation since in flat coated pits triskelions assemble into mostly hexagons and a few pentagon-heptagon pairs, but within highly curved coated pits a significantly large number of extra single pentagons (unpaired with heptagons) are present (Heuser, 1980). Perhaps the most controversial aspect of both models is the assertion that clathrin lattice transformations are initiated only by reorganization of bonding arrangements at the edge of a lattice patch. The difficulty with this assumption is that large extended patches of assembled flat hexagonal lattice often are observed under normal physiological conditions (Heuser, 1980, 1989) and, due to their size, the propagation of structural transformations from edges to interior is unlikely to be an essential step via which flat patches evolve into highly curved precursors of coated vesicles.

It also has been suggested that the cell membrane and its bound receptors are the primary initiator of curvature, while clathrin triskelions assemble passively along the resultant membrane contours (Heuser, 1980; Pearse and Crowther, 1987). However, under conditions such as low pH which especially favor coated-pit invagination, multiple-curved domains and invaginated coated pits with long necks (see Fig. 3) are observed (Heuser, 1980; Pastan and Willingham, 1985; Heuser, 1989; Lin et al., 1992). Although membrane mechanisms such as an asymmetric expansion of monolayers and lipid domain formations can induce curvatures and vesiculation (Forge and Devaux, 1992; Lipowsky, 1993), those vesicles and curving domains typically are of larger sizes. Furthermore, recent cell-free experiments (Moore et al., 1989; Lin et al., 1992) designed to reconstitute receptor-mediated endocytosis explicitly demonstrate that, so long as the clathrin lattice is absent, even membrane patches with clustered receptors do *not* invaginate. Vesicle formations from Golgi membrane does not involve clathrin coats; nevertheless, the budding of intercisternal transport vesicles requires another form of protein coat, the major component of which is named *coatomer* (Water et al., 1991; Orci et al., 1991). Consequently, it is hard to argue that membrane forces are the main causes of the those small, multiple-curved domains and narrow long necks observed during the budding of coated vesicles.

In fact, the experimental evidence discussed above strengthens a long-standing suggestion (Kanaseki and Kadota, 1969) that clathrin induces curvature directly into the interior of a coated pit via some *internal* lattice rearrangements that yield the needed pentagonal lattice facets. That is, triskelion energetics and interactions, influenced by the physiological environment, cause coated pits to invaginate and to bud off near the center of flat hexagon lattices. However, to firmly establish this thesis one must specify the sequence of steps by which the desired lattice transformations may occur. Since the invagination and budding-off are observed to proceed essentially spontaneously (Pastan and Willingham, 1985; Brodsky, 1988), each of these steps should be a local event involving relatively few rearrangements of triskelion bonds. Impeded by complicated packing structures of the clathrin lattices, researchers have not yet been able to find such suitable internal lattice transformations, a fact that prevents the universal acceptance of this mechanism over the competing ones discussed above (Schmid, 1992). Indeed, Heuser (1989) concluded that "... [it] remains a formidable challenge to explain how a lattice of the complexity of clathrin could rearrange bonds internally, as it would have to do if it were to convert the flat hexagons of a planar array into puckered pentagons needed for a curved array."

In the main body of this article we propose specific schemes of triskelion lattice transformation that, we believe, solve this puzzle concerning the mechanism of coated-vesicle formation. These schemes are uncovered and proven to be valid by using mathematical topological rules that govern all clathrin-like lattices.

C. Outline

Material presented in section II illustrates the fact that *additions* of *pairs* of clathrin triskelions to and about the center of an existing flat lattice patch can lead to the observed coated-pit invagination. Subsequently, we show that triske-

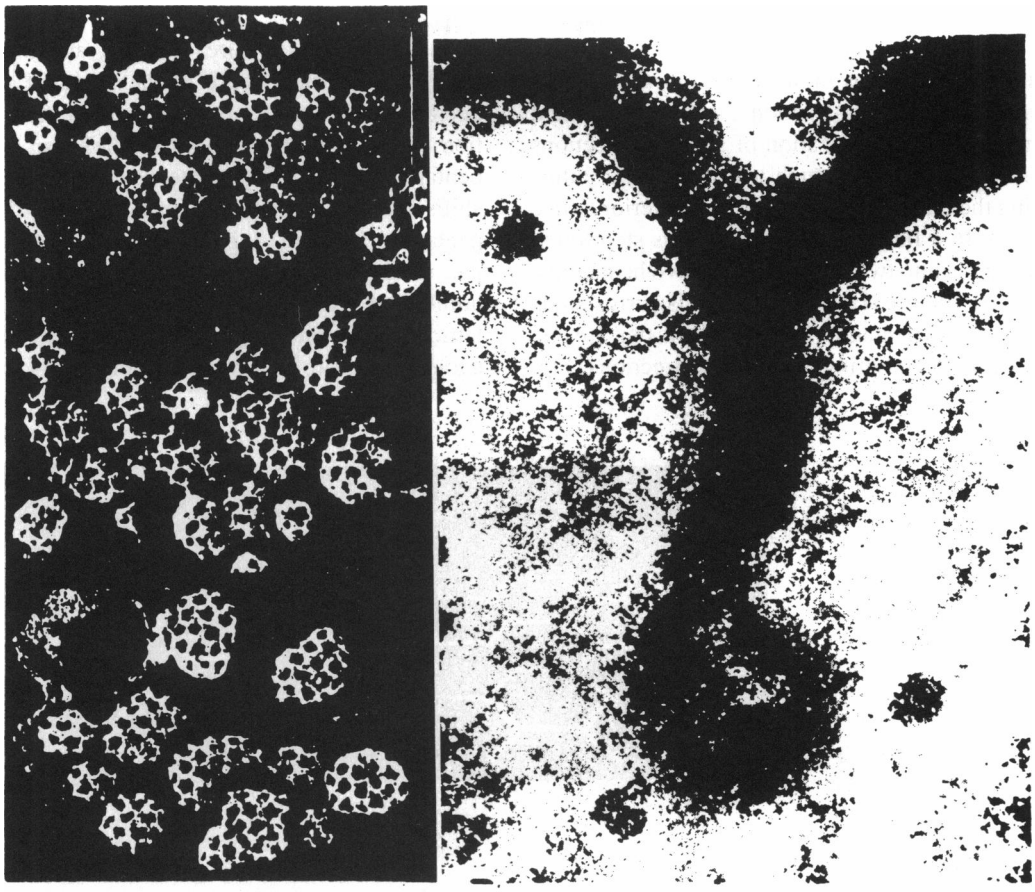


FIGURE 3 Examples of clathrin lattices with (*a*) multiply invaginated subdomains developed from large, initially flat hexagonal patches (taken from Heuser (1980), where the *vertical white bar* measures 2000 Å) and (*b*) a highly elongated, narrow pit neck (taken from Pastan and Willingham (1985)). Note that the diameters of most necks are only about 300 Å, while their length may exceed 10,000 Å. Internal lattice transformation is the only effective mechanism by which lattice curvature may increase beyond these stages.

lions may be removed, also in pairs, from the interior of the clathrin lattices without disrupting proper clathrin lattice connections. When these two processes are combined, vesicles of varying sizes and shapes are shown to be generated by changes which are confined totally to the interior of initially flat coated pits.

Section III contains essential derivations and discussion that justify the schemes described in section II. We carefully ascertain several rather simple and general topological rules governing clathrin lattices and their transformations. Based on the specific characteristics of clathrin lattices, these rules are *strict* mathematical constraints on the numbers of non-hexagonal clathrin lattice facets, and on the changes that might occur in these numbers during desired lattice transformations. This section establishes the uniqueness of the dimer addition/removal processes demonstrated in section II, and therefore is fundamental. Some supportive mathematical arguments are given in the Appendix, which may be skipped by readers who are not interested in such details.

Several implications of the proposed, modified lattice transformation mechanism are explained in section IV. We advance a minimal model for receptor-mediated endocytosis where clathrin triskelion energetics and interactions predominantly control internal lattice transformations which drive coated-pit invagination and coated-vesicle budding. Other factors, including the role of membrane forces and fusion, also are considered and briefly discussed.

II. SPECIFIC TRISKELION REARRANGEMENTS OF COATED-VESICLE FORMATION

A. The basic step of internal lattice transformation

As mentioned earlier, in order to understand fully coated-pit invagination and coated-vesicle budding, one needs to discern the mechanisms which allow desired clathrin-lattice shape transformations. In the following illustrations we demonstrate that the key steps of coated-pit invagination are associated with direct additions of pairs of triskelions from the cytoplasm to the center of a lattice patch.

The most distinct feature of all such invagination steps is that two new triskelion molecules are inserted into a lattice patch at each step. These two molecules form a triskelion *dimer* by overlapping their arm sections, and are integrated into the patch after the reorganization of a few connections with neighboring triskelions in such a way that the lattice bond connecting the dimer crosses an existing interior lattice facet (see Fig. 4). In a flat hexagonal array, this step transforms three hexagons into two pentagons and two heptagons. In terms of lattice structure (Heuser, 1980), this corresponds to the generation of two pentagon-heptagon pairs that are one lattice spacing apart and pointing in opposite directions (Fig. 4, *c* and *d*). On the other hand, in a curved lattice patch nonhexagonal facets already exist, and the insertion of a triskelion pair near those nonhexagonal facets produces a decreased amount of nonhexagons. Furthermore, the addition of a triskelion pair across a hexagon that bridges two pentagons does not generate new nonhexagons; it merely shifts the locations (and orientations) of the two existing pentagons (see Fig. 4, *e* and *f*). The addition of a triskelion pair during any invagination step always increases the total number of lattice faces by one: in the above case of flat lattice, four nonhexagonal facets are created, while three hexagonal facets are destroyed (see Fig. 4, *a–d*); in the curved-

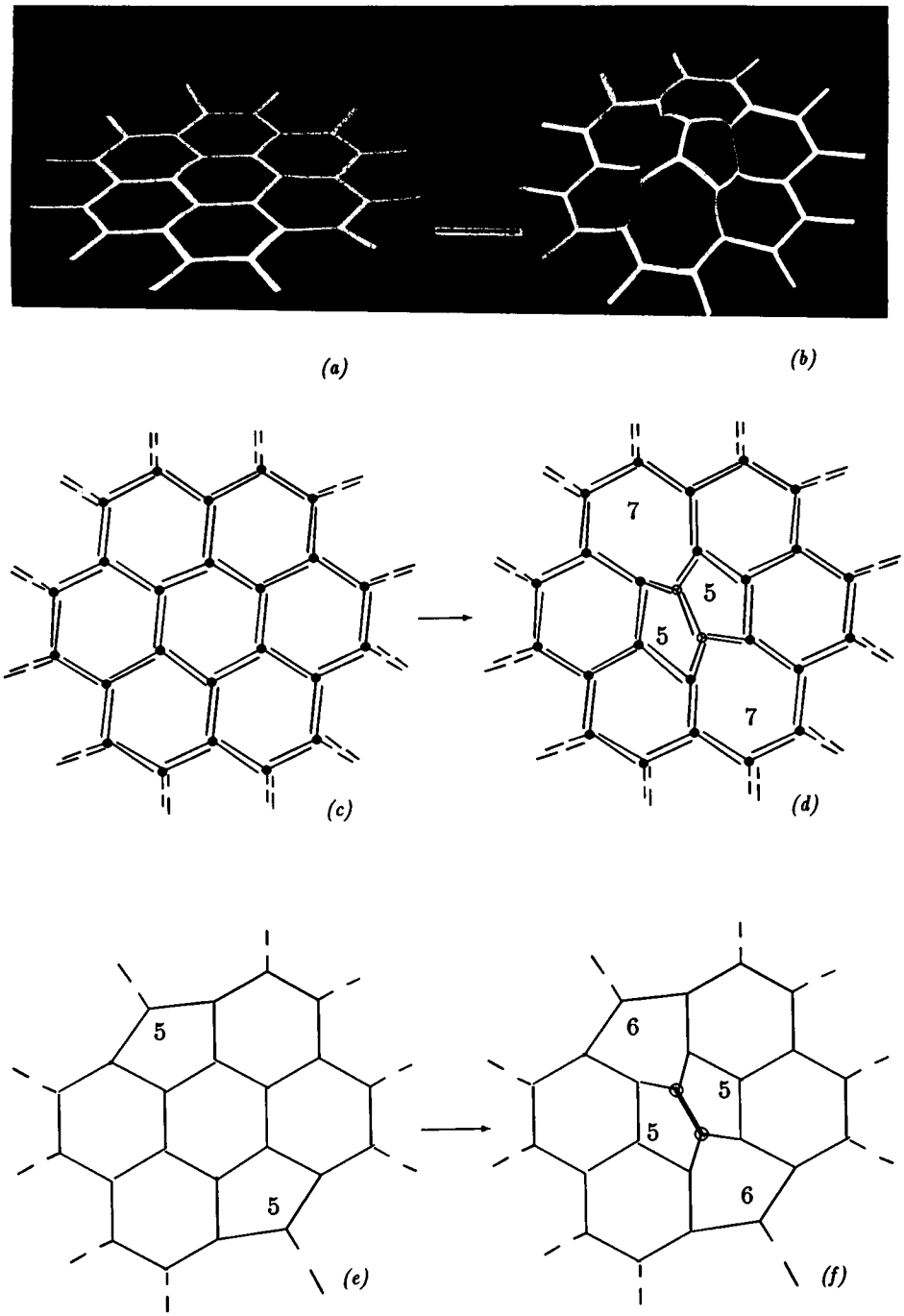


FIGURE 4 Illustrations of basic steps of internal lattice transformation. (*a* and *b*) Photographs of paper models of clathrin lattices indicating realistic coated-pit curvature occurring when two new triskelions are added to the interior of a flat hexagon array, creating two oppositely directed pentagon-heptagon pairs. (*c* and *d*) Drawings of the same lattice transformation using triskelion "monomers" of Fig. 1 *d* to illustrate the bond rearrangements and polygonal facet conversions. (*e* and *f*) Skeleton drawings of another dimer assembly transformation process, where the addition of the triskelion pairs converts two existing pentagons to hexagons and results in two new pentagons. The newly assembled triskelion dimers, identified by open circles in *d*, *f*, and later figures, always cause invagination and growth of the clathrin coat around the point of insertion. The arrows denote the completion of a given internal transformation. The double bonds in *f*, as well as in later figures, are used to highlight the inter-connection between the two newly inserted triskelions (as a dimer) (cf. Fig. 2). The numbers, 5, 6, 7, etc., mark some relevant polygonal facets.

lattice example, one new hexagonal face alone is created (see Fig. 4, *e* and *f*). Concurrently, each dimer insertion involves a net increase of three polygonal lattice bonds, consisting of overlapping triskelion arm sections (see Fig. 4, *c–f*).

Increases in numbers of facets and lattice bonds, together with the fact that realistic clathrin coats have rather uniform lattice-bond lengths (as discussed in section I.B), imply an increase of lattice surface area near the point of dimer insertion. To accommodate such increased lattice surface area, the lattice must undergo elastic relaxation which generates increased local curvature, given any reasonable triskelion intramolecular energetics and intermolecular interactions (see section I.A). In particular, as demonstrated by paper models in Fig. 4, *a* and *b*, a single dimer insertion to the interior of a completely flat hexagon array can induce a stable lattice having characteristics of a shallow coated-pit. Notice that the arrangement of the two pentagon-heptagon pairs in Fig. 4, *b* and *d*, assures the absence of any shape change of the hexagonal array far away from the newly assembled triskelion dimer. Similarly, in the case shown in Fig. 4, *e* and *f*, no new distortion of the lattice is induced except near the inserted dimer. In other words, inserting a pair of triskelions to the interior of the clathrin lattice of a coated pit always causes it to become a bit more deeply invaginated.

B. The formation of a hexagonal barrel

Combinations of these individual invagination steps together constitute the primary mechanism for triskelion-lattice transformations leading to coated-vesicle formation. We turn first to an explicit example in which a coated-vesicle with a *hexagonal barrel coat* grows out from the center of a flat clathrin pit. In the following, one may regard the generation of a vesicle with a hexagonal barrel coat as a three stage process: I) the generation of a "seed" structure; II) subsequent deep invagination; III) vesicle budding.

We illustrate the notion of the generation of the "seed" structure by Fig. 5. This compact seed, consisting of an inner ring of six pentagons and an outer ring of six heptagons in an otherwise hexagonal lattice, can be created from a flat lattice in six basic steps. The sequence indicated by Fig. 5, *a–e*, involves the addition of triskelion dimers across hexagonal, heptagonal, and octagonal (i.e., eight-sided) faces. As indicated, this sequence effectively turns the first ring of six hexagons surrounding the center hexagon in the original flat array into six *hept*agons, simultaneously creating six new pentagonal faces. In a real endocytotic process, this seed would appear on the cytoplasmic side of the cell membrane as a highly curved, hemispherical coated pit with all pentagons nearly perpendicular to the hexagonal face (see Fig. 5, *f*).

It has been concluded (Pastan and Willingham, 1985; Brodsky, 1988; Schmid, 1992) that subsequent deep invagination is associated with the growth of a long, narrow "neck," connecting the nascent vesicle to the remainder of the pit (see Fig. 3). With our mechanism, this neck may be generated and elongated from the compact seed via addition of triskelions in another six-step process. As illustrated in Fig. 6, *a–e*, the first section of the neck is generated by assembling six triskelion dimers around the center hexagon of the seed. It is formed by an encircling ring of six hexagons which are converted from the corresponding six pentagons in the seed. The neck then is elongated, section by section, by successive sets of these six basic triskelion addition steps. This process can produce deeply invaginated coated pits that are connected to the plasma membrane through very long necks before vesicle budding occurs (see Fig. 3).

A full understanding of the budding process cannot be achieved until the concurrent *membrane fusion* event, which probably is highly dependent on biological factors such as the presence of membrane-bound receptors and specific cytoplasmic conditions, is elucidated (Alberts et al., 1989; Schmid, 1992). This fusion event, which physically separates the would-be vesicle membrane from the cell plasma membrane beneath the clathrin coat, currently is a subject of intense investigation (Lin et al., 1992; Smythe et al., 1989; Schmid and Smythe, 1991). We observe, though, that the tight spatial confinement of the plasma membrane in the region of the narrow neck undoubtedly helps membrane fusion to occur in that region.

Once a fusion event leaves a certain amount of membrane at the bottom of a deeply invaginated pit through the above two stages, additional clathrin lattice transformations must take place to finally free a coated vesicle. Since in a normal endocytotic event triskelion addition is mediated by membrane surface, one can presume that triskelion bonds not supported by underlying membrane are greatly weakened (McKinley, 1983). As a result we expect that one or more ring of triskelions dissolves back into the cytoplasm, breaking the clathrin neck. Furthermore, based on the topological principles governing coated-pit invagination that are explained in the next section, we believe that the final detachment of a coated vesicle probably occurs by a *reversal* of the basic triskelion insertion step. That is, at each transformation a triskelion dimer is *removed* from the lattice by rearrangement of a few neighboring bonds. Returning to our explicit example, membrane detachment first occurs in the coated pit having a neck of four sections in length (see Fig. 6 *f*); then, a combination of these dimer removal steps, as illustrated in Fig. 7, *a–d*, releases the desired coated vesicle with a hexagon barrel coat having eight hexagonal faces and 12 pentagonal faces (see Fig. 7 *d*).

C. Further comments on lattice transformations

Several additional points need to be clarified. First, as already implied in the previous subsection, what we call a "seed" simply is a partially invaginated coated pit. Once this stage occurs, passage through the next two stages yields a coated vesicle and, in most cases, leaves a new functional seed since residual nonhexagonal facets usually remain in the pit. Therefore one can normally expect a stream of coated vesicles to grow out of any site where such a seed structure is located. Consequently, referring to these partially invagi-

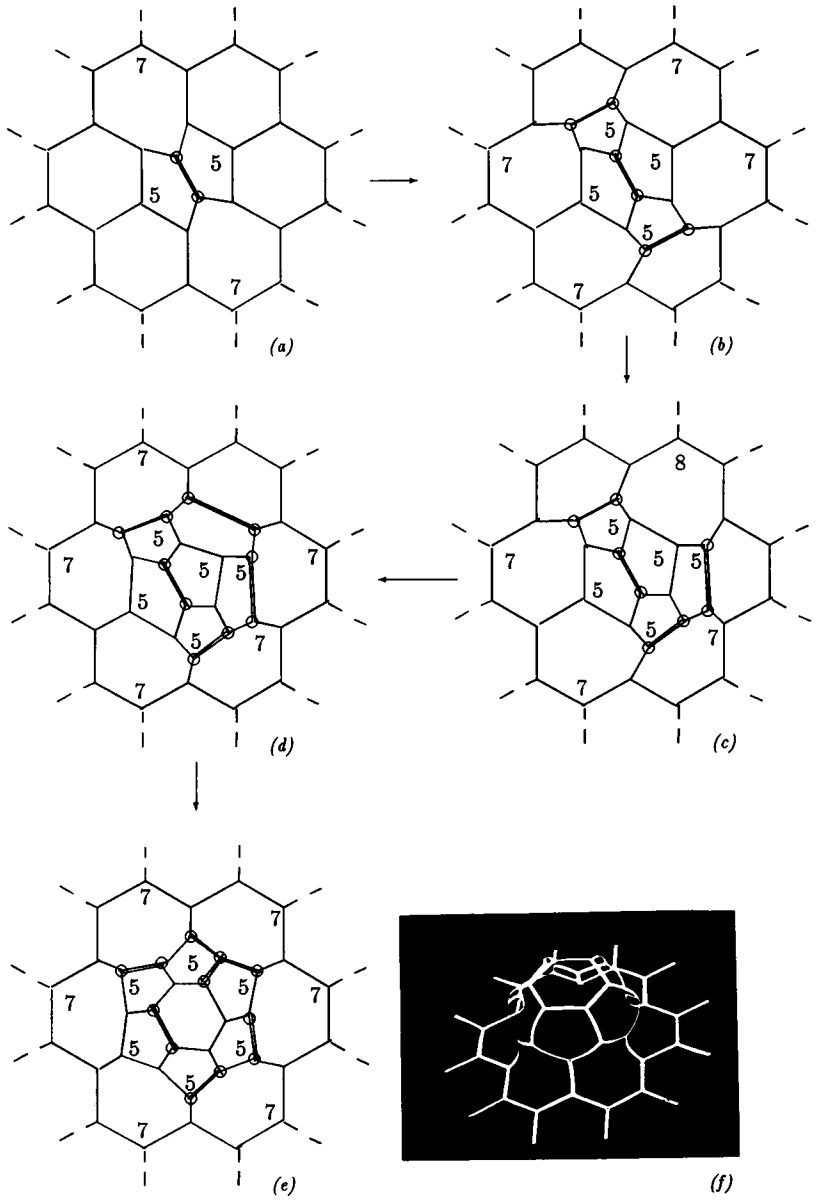


FIGURE 5 The generation of a "seed" structure (a semispherical coated pit) via basic assembly steps is illustrated: parts *a–e* show how dimers may be successively added onto the initially flat lattice array; *f* displays the highly curved spatial structure of this seed-in particular, the components of the ring of pentagons make rather large tilt angles with the plane of hexagons. One can directly verify that, for each transformation step shown in *a–e*, all the topological constraints discussed in section III are satisfied. In two-dimensional electron micrographs, the ring of heptagons may be quite difficult to distinguish from the hexagons.

nated coated pits as seed structures seems to be appropriate and informative. Note that, for energetic reasons, a typical seed structure likely is less compact (i.e., more gradually curved) than the example shown in Fig. 5 *f*.

Vesicle generation is shown in subsection II.B as starting from a completely flat hexagonal array. However, most coated vesicles probably originate from some previously invaginated coated pits. A few pure pentagons may well have been incorporated into a clathrin patch at the initial assembly before invagination starts. It also is possible that a few heptagons generated by internal dimer insertions are turned into hexagons or dissolved into free triskelions at the edge of lattice patches. Both situations would allow existence of seed structures with more pentagonal faces than heptagonal faces. In addition, our illustrations are presented as a linear sequence of events; in reality many basic assembly and disassembly steps may happen concurrently and in varying order. For instance, while the clathrin lattice is detaching itself around the neck region, triskelion dimer insertion/removal steps may occur in the nascent vesicle, to mitigate the stress involved in closure of the coat around the pinched area.

In such process of vesicle formation each seed structure can generate coated vesicles of many sizes and shapes. We show in Fig. 8, *a–i*, that, when increased numbers of triskelions are added via the series of basic internal lattice transformation steps to form increasingly large deep pits, the vesicles that are produced are likely to have increased overall sizes as well.

We emphasize that the specified internal triskelion-dimer insertion (i.e., assembly) process and its reversal (namely the triskelion-dimer removal or *dis*assembly step shown Fig. 7) provide the means for closed vesicles to quickly change their shapes without disassembling and reassembling large portions of their coats. For instance, if a coated vesicle initially is produced with a fairly rough and peaked clathrin coat, it can quickly become much smoother by losing a few triske-

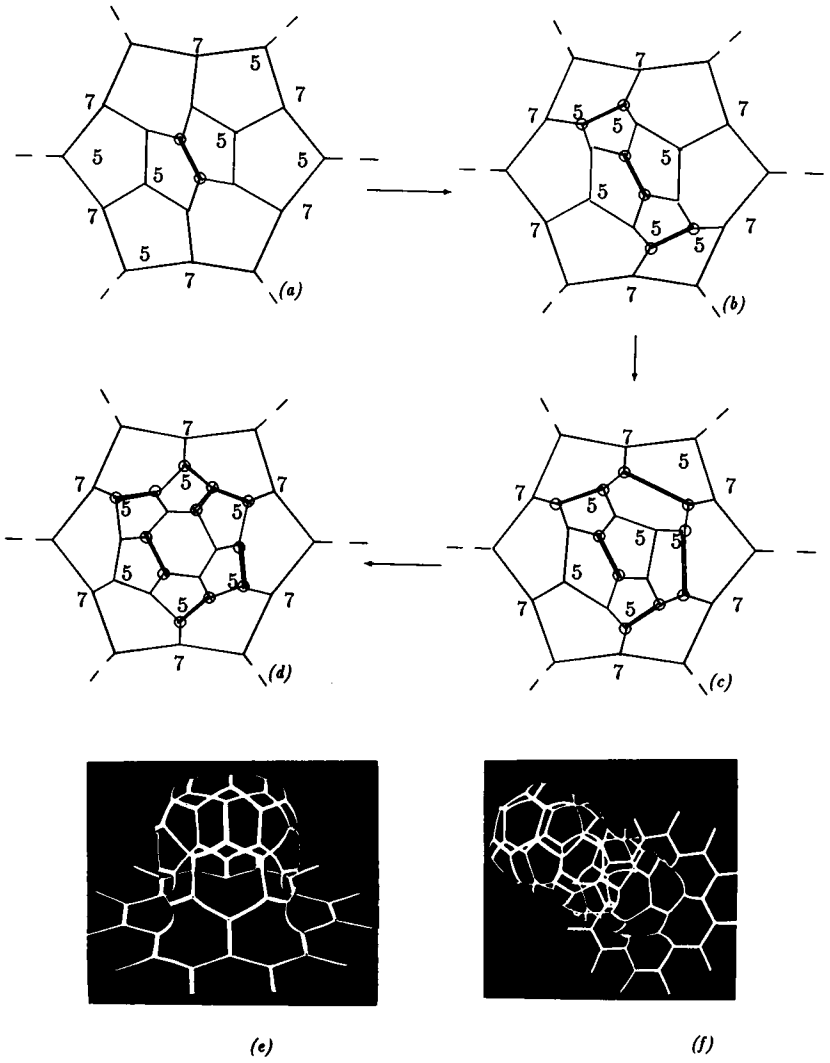


FIGURE 6 Continued growth of an invaginated coated pit by internal assembly of triskelion pair. Parts *a–d* show how the 1st to 6th pairs of dimers are added onto the polygonal facets of the "seed" generated in Fig. 5 to grow a pit neck of one section in length; *e* is the paper model version of *d* and, hence, is more representative of the real spatial structure of this coated pit; *f* displays, as an example, a pit with four neck sections. Each repetition of the assembly steps (*a–d*) successively increases the length of the pit neck.

lion dimers from the protruding peak regions and adding a few triskelion dimers onto some of the valleys. With such a mechanism a sufficiently large, elongated coated vesicle can split into two smaller and more spherical vesicles—without large-scale change of the clathrin coat. Because the baskets and nets are so highly interconnected, they previously were construed to be very resistive of such structure changes by other investigators (Heuser and Anderson, 1989; Pearse and Crowther, 1987; McKinley, 1983). The mechanism of internal lattice transformation that we are proposing effectively allows a degree of "plasticity" for triskelion lattices.

The "rigidity" of a specific clathrin net also is of interest. In order for a lattices to be topologically rigid, either it or its *dual* structure, has to be triangulated (Leob, 1976); of itself, large-scale connectivity of a space structure is insufficient. Because each internal triskelion is connected to exactly three neighboring triskelions, clathrin lattices are indeed the duals of triangulated structures and, hence, they are rigid. Consequently, clathrin coats at each step of invagination and in vesicles are capable of maintaining their shapes and forcing the curvature of underlying membranes. This rigidity of triskelion lattices is well illustrated by the paper models shown in Figs. 4–8.

The preceding discussion focuses on describing the details of a scheme of lattice transformation that leads to the creation of a coated vesicle formed from the interior of a flat coated pit. Ultimately, though, energetics determine why endocytosis occurs under suitable conditions. Based on the intramolecular energetics discussed in the Introduction, we believe that the properties of the triskelion naturally favor the described lattice transformations. For instance, the localization of shape change following each basic dimer-insertion (mentioned in section II.A) implies that relatively little elastic energy is involved during any given step. Hence, the free energy reduction owing to the formation of three new lattice bonds may easily overwhelm the elastic energy change, which can be either favorable or unfavorable depending on detailed intratriskelion energetics (see section I.A). Consequently, interior dimer-insertion should be a thermodynamically easy step.

Finally, we note that a single triskelion molecule might be momentarily washed out of its interior vertex position in a lattice patch since the attractive affiliation between triskelion arms should be finite. However, the resulting lattice would be defective; if such a lattice, having incomplete internal lattice connections, is not stabilized by the removal of an-

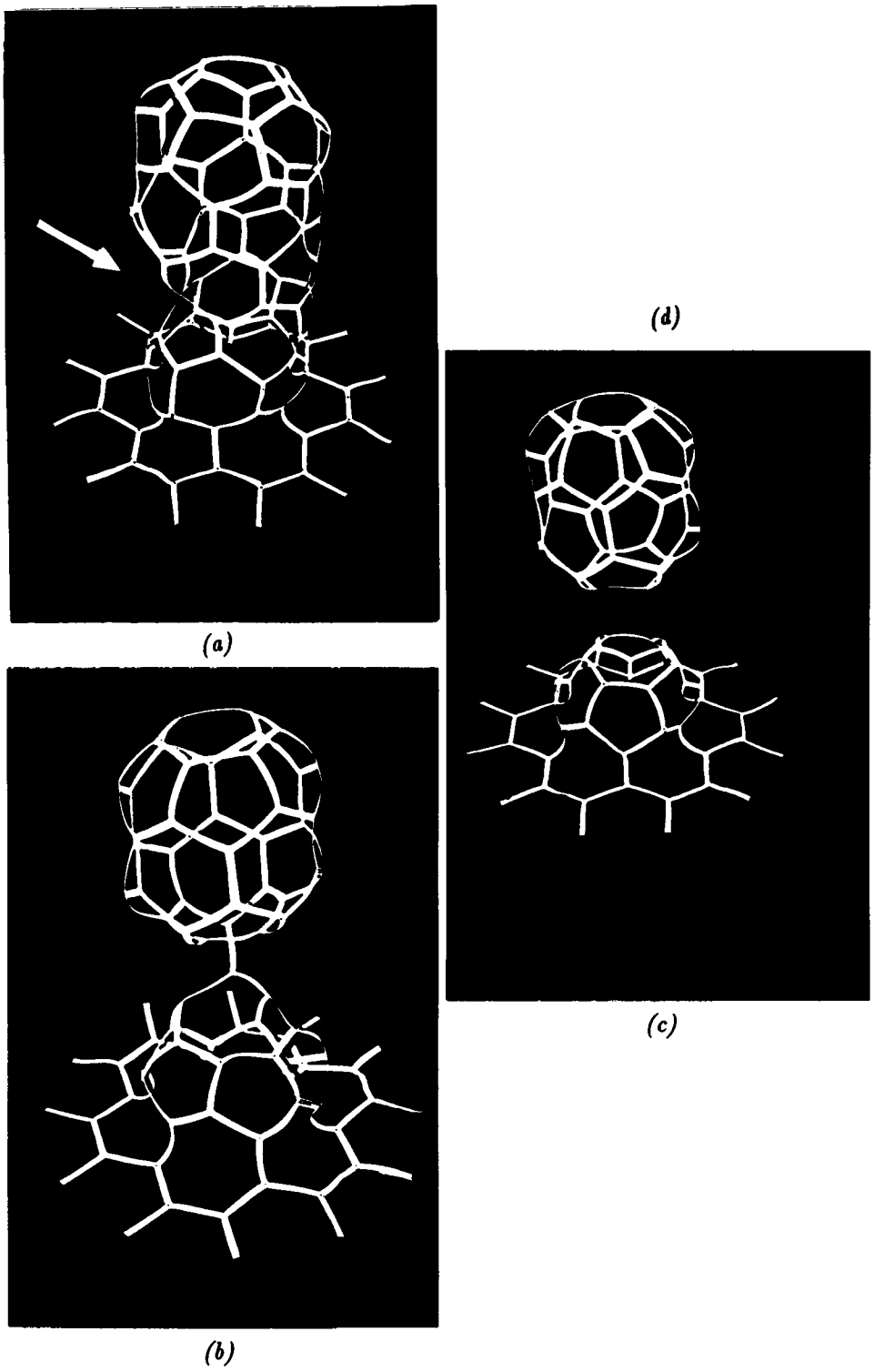


FIGURE 7 The budding of a free vesicle with a "hexagon barrel coat." Following a presumed membrane fusion event that detaches the plasma membrane underlying the second neck section of the coated pit shown in Fig. 6 *f*, the corresponding six pairs of triskelions without membrane support probably dissolve back into the cytoplasm in steps of dimer disassembly. The removal of the first triskelion pair results in a lattice with an eight-sided facet (*white arrow*), as illustrated in Fig. 7 *a*. After five triskelion pairs are removed to reach the structure shown in Fig. 7 *b*, this facet effectively becomes a 16-sided polygon. Parts *c* and *d* show the hexagon barrel vesicle detached from the residual seed after the last triskelion pair is removed. In order to count the nonhexagons properly in accord with Eq. 2, one has to include the facet bounded by *both* the six edges of the center hexagon of the seed (*c*) *and* the six edges of the bottom hexagon in the "hexagon barrel coat" (*d*) as an 18-sided polygon. Since such a facet normally is ignored, one may regard the last lattice-detaching, dimer removal step as accompanied by the destruction of an 18-sided polygon. This finally creates 12 pure pentagons, without any ($n > 6$)-agons as counterparts out of the original seed where only pentagon-heptagon pairs exist. After the vesicle with the hexagon barrel coat moves to the interior of the cell, the residual functional seed structure may generate more vesicles of (usually) different sizes and shapes (see Fig. 8).

other triskelion molecule so that a basic dimer removal step is formed, the defective vertex would be quickly repaired by attracting another triskelion molecule from the cytoplasm. Likewise, a single triskelion can be momentarily linked to an interior triskelion, but stable lattice transformation occurs only if a dimer insertion step subsequently is consummated. Therefore, we always restrict our discussion to "complete" clathrin lattices and their shape transformations. The term "complete lattice" signifies that every internal triskelion is properly connected to exactly three neighboring triskelions, as is generally observed (Pastan and Willingham, 1985; Crowther and Pearse, 1981; Pearse and Bretscher, 1981).

III. TOPOLOGY OF TRISKELION LATTICE TRANSFORMATION

We now mathematically justify the steps proposed in the last section for coated-pit invagination and subsequent lattice

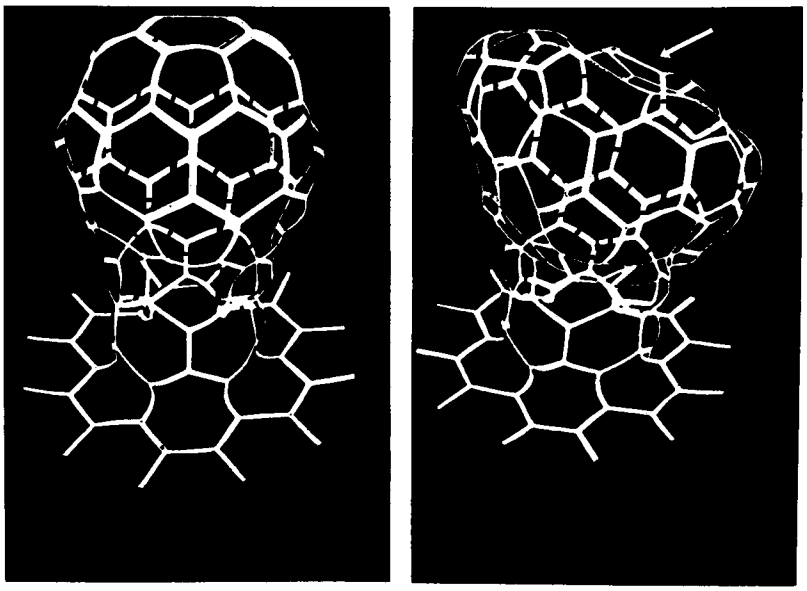


FIGURE 8 Paper models showing deeply invaginated coated pits on the verge of releasing coated vesicles of different sizes and shapes. (*a*) A spherical vesicle with "soccer ball" coat having 60 triskelions forming 12 pentagons and 20 hexagons; (*b*) an aspherical, larger coated vesicle having one heptagonal facets (*top front* and designated by the *white arrow*), 13 pentagons and numerous hexagons. To aid the viewer we have marked, with narrow gaps, the bonds that are on the far side of the structures.

transformation. We first show that the insertion of a triskelion dimer is, in fact, the simplest possible internal lattice rearrangement that increases the curvature of a clathrin network while preserving essential integrity. Most related observations then follow naturally.

What do we mean by referring to the "essential integrity" of a clathrin lattice? In general, this term simply denotes the basic characteristics of the coat structure that we have discussed in the Introduction and at the end of the previous section. Most importantly, in a complete triskelion lattice each interior vertex is connected exactly to three neighboring vertices, reflecting the three-armed structure of individual triskelions (see Fig. 1); furthermore, no triskelion "bond" (i.e., connection between two vertices) crosses another (see Fig. 2). In somewhat more mathematical terms, these properties describe the *topology* of the clathrin lattices when viewed as graphs that consist of lines (the "bonds") and dots (the vertices). In particular, the above-mentioned characteristic ensures that complete clathrin lattices correspond to *planar graphs* (Leob, 1976; Trudeau, 1976; Nossal and Lecar, 1991). Consequently, some very general topological rules will apply to the coat structures of all pits and vesicles, and to structural transformations.

A. Euler's Formula

The most fundamental topological rule that governs planar graphs (and clathrin lattices) is *Euler's Formula* (Leob, 1976; Trudeau, 1976; Nossal and Lecar, 1991), which relates the total numbers of the vertices, V, of connections ("bonds" or edges), E, and of faces, F, according to

$$2 = V - E + F. \quad (1)$$

A rigorous proof of this relation can be found, for instance, in Loeb (1976). In the Appendix to this paper we show, as two examples, that both the closed hexagon barrel coat (see Fig. 7 *d*) and an open flat hexagonal array satisfy this formula.

B. Implications of Euler's Formula

Euler's Formula can be used in many ways to understand aspects of clathrin-lattice-like structures and their transformations. For instance, Euler's Formula has been used directly to explain why, if no other type of polygonal facets are present, exactly 12 pentagons but a variable number of hexagons are found in clathrin coats of different sizes and shapes (Kanaseki and Kadota, 1969; Heuser, 1980; Nossal and Lecar, 1991). (The hexagon barrel coat falls within this class.) However, to comprehend the lattice *transformations* that lead to endocytotic vesicles, one needs to provide the following generalizations that, we believe, have some novelty. Because pentagons and heptagons are often observed in micrographs of coated-pits in addition to hexagons and, in principle, other lattice polygons also are allowed, we have derived in the Appendix the following generalized constraint for clathrin coats of *closed* vesicles of all shapes and sizes,

$$12 = 3F_3 + 2F_4 + 1F_5 + 0F_6 + (-1)F_7 + (-2)F_8 + (-3)F_9 + \ldots, \quad (2)$$

where F_i denotes the number of i-sided ($i \geq 3$) lattice polygon facets that are present. This equation holds for any clathrin coat, due to the special topological characteristics of the unit triskelions as explained in the Appendix.

In principle, then, a clathrin coat need not have exactly 12 pentagons, even through this often may be the case. Specifically, $\{F_i\} = \{0, 6, 0, 0, \ldots\}$ satisfies Eq. 2, implying that a closed clathrin network may take the form of a cube with just six square facets (and eight triskelions, one at each vertex corner). Such clathrin cubes have indeed been observed un-

der special, nonphysiological conditions through membrane-free triskelion assembly (Sorger et al., 1986). Many other combinations of nonhexagonal facets satisfying Eq. 2 are possible and in each case the number of hexagonal facets is always unrestricted; for instance, two square polygons, nine pentagons, one heptagon, and an unrestricted number of hexagons can easily form a complete, closed clathrin vesicle coat (see also Fig. 8 *b*).

Similar topological considerations have further important implications for internal lattice transformations. Specifically, since Euler's Formula, Eq. 1, applies to a lattice patch both before and after a given transformation, it follows that

$$0 = \Delta F - \Delta E + \Delta V, \tag{3}$$

where $\Delta X \equiv (X \text{ after the transformation} - X \text{ before the transformation})$. Although the patch of clathrin lattice must remain interconnected, it can either be the coat of a closed vesicle or that of an open pit. Moreover, if bond rearrangements do not involve the edge of a lattice patch (and, of course, if they preserve the basic triskelion lattice characteristic), Eq. 2 imposes the new constraint,

$$0 = 3\Delta F_3 + 2\Delta F_4 + \Delta F_5 - \Delta F_7 - 2\Delta F_8 - 3\Delta F_9 + \ldots. \tag{4}$$

In other words, for the kinds of *internal* lattice transformations that we are looking for, both Eqs. 3 and 4 hold. In much the same spirit, one finds (see Appendix) that

$$\Delta E = \frac{3}{2}\Delta V, \tag{5}$$

which allows us to derive, from Eq. 3, another simple and useful relationship,

$$\Delta F = \frac{1}{2}\Delta V. \tag{6}$$

Let us now clarify what specifically the topological relations given by Eqs. 4–6 imply. A direct consequence of Eq. 4 is that no pentagons may be generated internally without the simultaneous creation of heptagons and/or other ($n > 6$)-agons. This fact should be of particular interest to workers who have searched unsuccessfully for an internal mechanism that merely turns hexagons directly into pure pentagons. Indeed, as pointed out previously (Heuser, 1980; Pearse and Crowther, 1987), to turn one interior hexagon into a pentagon would require a major rearrangement of the lattice, a procedure that is very inefficient and almost impossible to realize under usual endocytotic conditions.

Given that all lattice bonds have a length similar to that of the proximal arm section of a clathrin triskelion (see Fig. 1), Eq. 4 indicates that internal lattice transformations essentially preserve the average area of the individual polygonal facets of a lattice patch. On the other hand, for coated-pit invagination to proceed, the total lattice area near the center of the pit must increase. Consequently, the total number of facets in the center region must increase as a pit deepens and its bottom enlarges. Equation 4 readily allows for a change in total number of facets, since it does not restrict the number of hexagons (i.e., F_6).

Equations 5 and 6 here have special relevance. Recall that only one triskelion lies at each vertex, so the number of vertices equals the number of triskelions assembled into the lattice. Thus Eq. 6 simply says that for each net increase of a polygonal facet, two triskelions must be added to the center region of an invaginated coated pit. Equation 5 concurrently assures that there will be a net increase of three lattice bonds for each newly added triskelion pair. Evidently, this internal addition of two triskelion molecules as a dimer unit corresponds precisely to the basic assembly step emphasized in section II for coated-pit invaginations.

More generally, the above results also justify the dimer-disassembly steps used for vesicle budding and clathrin lattice transformations in coated vesicles. In order that the basic characteristics and the implied rigidity of the clathrin coat be maintained, triskelions may be removed internally only in pairs. Each dimer disassembly reduces the total number of lattice facets by one and the total number of internal connections (lattice bonds) by three.

The discussed topological rules, especially as given by Eq. 4, explain why all the pentagonal facets observed in the illustrations shown in section II have heptagonal, or other ($n > 6$)-agonal, partners. However, applying these relations to the final step of vesicle budding requires extra care when lattice-patch splitting, as exemplified and further explained in Fig. 7, *c* and *d*, occurs. By treating released coated vesicles and their residual seed structure as independent entities, one finally observes more ($n < 6$)-agons than ($n > 6$)-agons in the system at the moment of pinching off. For each coated vesicle produced, the numbers of increased nonhexagonal facets satisfies Eq. 4. Of course, pentagons and other ($n \neq 6$)-agons are destroyed when complete triskelion decoating of internalized vesicles takes place at appropriate point during the endocytotic cycle.

IV. DISCUSSION

We have shown that topological considerations unambiguously require that internal clathrin lattice transformations proceed via basic assembly and disassembly steps in which *pairs* of triskelion molecules are added or removed from interior regions of the lattice. The examples discussed in section II indicate that combinations of such steps indeed can cause flat clathrin-coated pits to become invaginated and grow long necks through a progression of triskelion additions. Similar schemes allow coated vesicles to bud off, and to change their shapes permanently and easily after they are already in the interior of a cell.

The notion that coated-pit invagination proceeds in concert with internal clathrin bond rearrangement of the lattices (Kanaseki and Kadota, 1969; Heuser, 1980; Sanan and Anderson, 1991; Lin et al., 1991) finally is substantiated. Our findings emphasize two additional factors: the net acquisition of free triskelions from the cytoplasm and the impossibility of turning hexagonal facets directly into pure pentagons. The

neglect of the above two factors seems to be the main reason why previous searches (Heuser, 1980; McKinley, 1983; Heuser, 1989) for this internal mechanism have failed.

By strengthening the role of lattice transformation, our findings eliminate the need to involve membrane forces as being mainly responsible for introducing coated-pit curvature and coated-vesicle formation (Heuser, 1980; Pearse and Crowther, 1987). As detailed in section I.B, membrane mechanisms are not supported by recent cell-free experiments (Moore et al., 1989; Lin et al., 1991; Orci et al., 1993). These experiments explicitly demonstrate that coat proteins including triskelions are by far the most important components mediating coated-vesicle formation under normal physiological conditions.

Our results also make the postulated pentagon-diffusion (Pearse and Bretscher, 1981; Pearse and Crowther, 1987) and kinetic treadmilling (McKinley, 1983) mechanisms for lattice transformations less likely. Both schemes rely solely on the creation of pentagons along the edges of lattice patches, yet topological relationships imply that their diffusion to other points in the lattice is *impossible* without the insertion and removal of triskelion pairs. That is, the total number of triskelions in the center region must increase for a coated pit to invaginate; migration of isolated pentagons is insufficient. The kinetic treadmilling mechanism (McKinley, 1983) would require large scale disassembly and reassembly of portions of clathrin lattice for any shape transformation that occurs near the center of a pit. Furthermore, neither scheme can have any relevance at later stages of pit invagination, where the amount of free lattice edge is small in relation to the size of the lattice, or in shape changes of existing coated vesicles.

Based on the above considerations, modifications of various aspects of the general picture for receptor-mediated endocytosis can be postulated as follows. Because internal lattice transformations involve only a few triskelion bonds and, hence, a small energy variation at each step, one can expect that the distributions of both coated pits and coated vesicles (with various sizes and shapes) would be near thermal equilibrium with the cytoplasmic pool of free triskelions. When conditions are favorable for endocytosis, two situations must be true: (*a*) the natural distribution of coated pits on cell membrane includes a large population of deeply invaginated structures having long and narrow necks; and (*b*) helped by the spatial confinement along the necks, membrane fusion (including the reattachment of vesicle membrane to the plasma membrane) happens at a rather high rate. Given *a* and *b*, lattice detachment transformations, as exemplified by Fig. 7, should take place because plasma membrane enhances triskelion-triskelion affiliation and assembly. Lattice detachment then irreversibly drives the generation of coated vesicles and leads to an overpopulation of coated vesicles in the cell cytoplasm. Consequently, net vesicle *de*coating occurs in order to reestablish equilibrium. Concurrently, due to the reduction in the number of deeply invaginated coated pits and a concomitant increase of free triskelions, the coated pits on the plasma membrane start to invaginate further through the proposed internal lattice assembly processes. This restores the population of deeply invaginated coated pits having long and narrow necks, which gives rise to the next generation of coated vesicles. Hence the circulation of triskelions continues and additional extracellular molecules enter the cell.

The important point is that clathrin lattice transformation is the critical element in coated-pit invagination, coated-pit budding, and coated-vesicle decoating. A thermal equilibrium model implies that no active energy source is required for these clathrin functions. The role of membrane, membrane-bound receptors, nucleotides (e.g., ATP) and other biological factors is secondary, mainly affecting the circulation of clathrin triskelions by (i) influencing the properties of triskelions, e.g., to increase the binding of triskelion arms along the plasma membrane, (ii) controlling membrane fusion along the necks of deeply invaginated pits, and/or (iii) maintaining cell organization, including a suitable distribution of triskelions throughout the cell. Although these secondary factors may result in conditions where the rate of clathrin-mediated endocytosis is negligible (because, for instance, deeply invaginated pits are not present and/or membrane fusion along the necks cannot readily occur), it is the clathrin triskelions that provide the *essential tendency* for endocytosis to occur as a result of their assembly/disassembly and lattice transformation properties.

Of course, to fully appreciate relevant issues, detailed energetic mechanisms of internal clathrin lattice transformations need to be expounded quantitatively (beyond the qualitative discussion in section II.C and elsewhere). It is important to substantiate that a reasonable model of effective triskelion energetics and interactions, such as for the idealized model shown in Fig. 1, may be advanced to describe coated pits and coated vesicles having experimentally observable behaviors and properties, including size and shape distributions. One also needs to understand how specific biochemical factors and variables, studied in recent assays (Heuser and Anderson, 1989; Sanan and Anderson, 1991; Lin et al., 1991; Lin et al., 1992; Smythe et al., 1989; Schmid and Smythe, 1991), influence the overall properties of triskelions and any underlying biochemical mechanisms.

For this purpose the application of thermodynamic and statistical principles for triskelion lattice transformation (Wegner, 1976; McKinley, 1983) will be important. The latter will involve accounting for a pool of free cytoplasmic triskelions. Knowledge of triskelion intramolecular energetics (Yoshmura et al., 1991; Kocsis et al., 1991) is crucial and mechanisms of membrane transformation (Huse and Leibler, 1988; Farge and Devaux, 1992; Lipowsky, 1993) may play a quantitatively important role. Membrane-bound receptor diffusion (Goldstein et al., 1984) and aspects of cytoplasmic composition also may be relevant when comparing postulated endocytotic mechanisms with experiments. These factors should be studied in parallel with the topological schemes presented in this paper, as the latter impose stringent constraints that need to be taken into account. However, such considerations are beyond the scope of this paper.

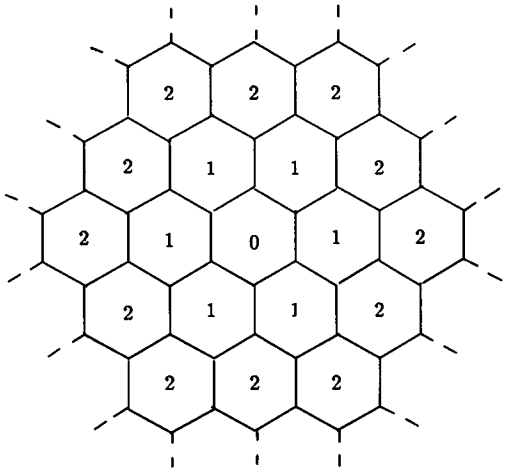


FIGURE 9 A flat, symmetric lattice patch that obeys Euler's Formula. After marking the center hexagon with a zero, all other hexagons belong to the first ring, the second ring, etc.

APPENDIX: PROOFS OF TOPOLOGICAL RELATIONS

We here illustrate that *Euler's Formula*, Eq. 1, is satisfied by the following two examples of clathrin lattices. (i) First, for the hexagon barrel coat, which corresponds to a *planar graph*, because it can be transformed into a flat structure without changing its topological character, a direct count yields $F = 20$ (there being 12 pentagonal faces plus eight hexagonal faces), $V = 36$, and $E = 54$. These numbers exactly satisfy Eq. 1. (ii) Next, we consider a symmetric lattice patch of any size (see Fig. 9). In this case, a more careful counting is necessary. After designating the center hexagon with the symbol "0," one specifies the size of the patch by the number of rings of hexagons that it contains (see Fig. 9). A patch containing only the 0th ring (i.e., only one hexagon) has $V^0 = 6$ vertices, $E^0 = 6$ bonds, and $F^0 = 2$ faces-one being the hexagon, the other being the surrounding face which extends to infinity. These numbers give $F - E + V = 2 - 6 + 6 = 2$ for the 0-ring patch. More generally, a k-ring patch with $k > 0$ can be analyzed by first noting that $f_i = 6i$ hexagons are present in any ith ring ($i \geq 1$), that $e_i = (3f_i + 6)$ bonds exist which separate these n_i hexagons from each other and from the $(i + 1)$st ring, and that $v_i = (2f_i + 6)$ vertices are located between the ith ring and the $(i + 1)$st ring. One next observes that the topological indices of the k-ring patch may be computed as

$$F^k \equiv F^0 + f_1 + f_2 + \ldots + f_i + \ldots + f_k$$
$$= 2 + 6(1 + \ldots + k) = 2 + 3k(k + 1), \quad \text{(A1)}$$

$$E^k \equiv E^0 + e_1 + e_2 + \ldots + e_i + \ldots + e_k$$
$$= 6 + 6k + 18(1 + \ldots + k) = 6 + 3k(3k + 5), \quad \text{(A2)}$$

and

$$V^k \equiv V^0 + v_1 + v_2 + \ldots + v_i + \ldots + v_k$$
$$= 6 + 6k + 12(1 + \ldots + k) = 6 + 6k(k + 2). \quad \text{(A3)}$$

Finally, one verifies that

$$F^k - E^k + V^k = 2, \quad \text{(A4)}$$

for any given value of k. Of course, to satisfy the Euler's Formula the lattice patch need not be symmetric.

In the remainder of this Appendix we derive Eqs. 2 and 5, using the specific characteristics of clathrin lattices. We first note that the total number of faces is given by

$$F = F_3 + F_4 + F_5 + \ldots + F_i + \ldots. \quad \text{(A5)}$$

Then, recalling the basic characteristics of clathrin networks, we notice that each vertex is always shared by three faces. Thus, given that each i-sided face shares i vertices, the total number of vertices is related to the $\{F_i\}$ as

$$V = \frac{1}{3}[3F_3 + 4F_4 + 5F_5 + 6F_6 + \ldots]. \quad \text{(A6)}$$

In the same spirit, each i-sided face takes part in i bonds (connections) and each bond is shared by two faces, so the total number of bonds is specified according to

$$E = \frac{1}{2}[3F_3 + 4F_4 + 5F_5 + 6F_6 + \ldots]. \quad \text{(A7)}$$

Consequently, after inserting Eqs. A5–A7 into Eq. 1, Euler's Formula takes the form

$$2 = \sum_{i \geq 3}\left[1 - \frac{i}{2} + \frac{i}{3}\right]F_i, \quad \text{(A8)}$$

which reduces to Eq. 2 upon simple rearrangement. Finally, given our counting system, one finds, from Eqs. A7 and A8, that $V = (2/3)E$ and, hence, obtains the expression given by Eq. 5.

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