

Physical Principles in the Construction of Regular Viruses

D. L. D. CASPAR AND A. KLUG*

*The Children's Cancer Research Foundation, The Children's Hospital Medical Center,
and the*

Department of Biophysics, Harvard Medical School, Boston, Massachusetts;

**Medical Research Council Laboratory of Molecular Biology, University Postgraduate Medical School, Cambridge, England.*

THE FUNCTIONAL ORGANIZATION OF VIRUS PARTICLES

There are two key facts about viruses from which all consideration of their structure and functional organization must proceed. The first is that the essential infective agent of all viruses is a high molecular weight nucleic acid component—either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Second, the nucleic acid molecule is contained in a protective package which serves to transmit this infectious agent in a functionally intact state through space and time to a susceptible host.

The virus nucleic acid has the capacity of re-directing the synthetic machinery of its host cell to the production of more virus. It is becoming increasingly clear that this control over the cell metabolism can be exerted at a number of different stages of normal biosynthesis. The DNA of large bacteriophages, for example, may pertinently be regarded as a transmissible piece of bacterial chromosome (Luria, 1959). In contrast, the RNA of tobacco mosaic virus and presumably of other RNA viruses, appears to be homologous to the normal messenger RNA of the cell (Matthaei et al., 1962). Indeed, the ultimate classification of many viruses may be primarily in terms of their relation to normal cell constituents.

It is not merely a matter of labeling viruses as DNA- or RNA-containing, but also of distinguishing them in terms of the amount of information carried by the nucleic acid. A complex DNA virus may be able to direct the synthesis of many new enzymes, as well as its own structure protein, whereas a simpler DNA virus may be able to specify only a small number of proteins. On the assumption of a universal coding ratio (Crick, Barnett, Brenner, and Watts-Tobin, 1961) between nucleic acid and protein, the amount of information transmitted by a virus would depend on the size of its nucleic acid moiety. Large DNA viruses contain several hundred times as much nucleic acid as the very small DNA and RNA viruses. The RNA of a small bacterial virus (Loeb and Zinder, 1961) consists of only about 1,600 nucleotides (molecular weight 500,000) which, if the cod-

ing ratio is 3:1, could specify at most only two or three different protein molecules. The comparably small tobacco necrosis virus particles (Kassanis and Nixon, 1960, 1961) do not appear to carry complete enough information for their own multiplication, and can only reproduce in association with another, larger tobacco necrosis virus. The molecular weight of the DNA content of vaccinia (Smadel and Hoagland, 1942) and *Tipula* iridescent virus (Thomas, 1961) are both about 150×10^6 , which is considerably greater than the DNA content of the small living cells of pleuro-pneumonia-like organisms (PPLO) (Morowitz et al., 1962).

The infectivity of a virus must persist in a latent state outside the host cell. Isolated nucleic acid molecules are very labile, particularly in an intercellular environment containing nucleases. If the virus is to succeed in propagating itself, its nucleic acid must be contained in a protective package. This is achieved by the provision of a protein coat or framework which contains the nucleic acid. It may appear, at first sight, that there is an enormous variety in the ways in which this could be done, judging, for instance, only by the range of morphological variation found in viruses. On the contrary, it is the main thesis of this paper that this is not so. The important point is that there are only a limited number of efficient designs possible for a biological container which can be constructed from a large number of identical protein molecules (Caspar and Klug, 1963). The two basic designs are helical tubes and icosahedral shells. For this reason, the same kind of molecular architecture may turn up in RNA or DNA viruses infecting animals, plants, and bacteria.

The structure of biologically completely unrelated viruses—for example, poliovirus and turnip yellow mosaic virus—may be based on very similar designs. Thus, the use of morphology or symmetry as a basis for classifying biological interrelationship must be regarded with caution. Although it is quite likely that closely related viruses will be morphologically similar, the converse is not true. A firmer basis for classification of biological relationship between viruses might be based on the more peripheral aspects of their structure (cf.

Franklin, 1962). Such aspects would include the presence of accessory components serving a special function, or related to the mode of reproduction.

SIMPLE, OR MINIMAL VIRUSES

Our current ideas on the structure of viruses are based on Crick and Watson's suggestion (1956) that all small viruses are built up of identical protein subunits packed together in a regular manner to provide a protective shell for the nucleic acid. A biological argument for construction out of subunits is that coding of the coat protein in the form of small identical molecules is an efficient use of the limited information contained in the virus nucleic acid (Crick and Watson, 1957). This hypothesis has been amply borne out in the case of a number of small viruses by X-ray diffraction (for a review, see Klug and Caspar, 1960) and by electron microscopy (see Horne and Wildy, 1961).

It has become increasingly clear that the term "small virus" used by Crick and Watson in the above connection is somewhat of a misnomer. The feature that should be stressed is that a virus, built up of identical subunits, will have a uniform size and regular shape. There are many large viruses which fall into this category—for example, the insect virus, *Tipula* iridescent virus, which has a (frozen-dried) diameter of about 1300 Å and has the shape of a regular icosahedron (Williams and Smith, 1958). Thus, while all small viruses appear to be regular, not all regular viruses are small. We propose to use the term simple virus for those viruses which have a regular particle structure and have as their main chemical components only nucleic acid and protein.

There are two main structural types of simple virus: (1) the rod-shaped particle which in its ideal form may be rigid, but which also includes the more flexible filamentous viruses; (2) the isometric particle, which has often been referred to as "spherical". As we shall see, the distinction between "spherical" or polyhedral shape is of no fundamental importance. Indeed, all known examples of isometric viruses have icosahedral symmetry, and such viruses may well be referred to in the future as icosahedral viruses.

The simple viruses are a select class and are by no means representative of the range of variation that is possible among viruses. They do, however, possess the fundamental properties of being able to reproduce within a living cell, and of being able to persist in an inert extracellular form until they encounter other cells they can infect. Detailed structural studies can be carried out on these viruses because of their relative simplicity. These studies are revealing the minimal properties of a

virus. Indeed, these simple infectious agents may be defined as minimal viruses. All viruses must possess at least these minimal properties and it is, therefore, not surprising that objects resembling simple viruses in structure have been found as constituents of more complex viruses, such as the myxo- and tumor viruses. These are the primary packages of the infectious nucleic acid, or, as we should now say, *nucleocapsids* (see Proposals, Caspar et al., this volume).

The myxoviruses contain in their interior, loosely-coiled, rope-like structures bearing a strong resemblance to that of the flexible, helical plant viruses (Horne et al., 1960; Hoyle, Horne, and Waterson, 1961). These structures correspond to the "soluble" nucleoprotein antigen and may be legitimately regarded as primary packages of the nucleic acid of the virus. There is now a good deal of evidence (see Bernhard, 1960) that many tumor viruses possess a dense nucleoid of about 400 Å in diameter. The simplest form reported is that of polyoma, which, in our terms, may be regarded as a bare primary package of nucleic acid. In the case of the mouse mammary tumor agent (Moore et al., 1958) it has been shown that the nucleoid itself (the "small" agent) is infective, but less stable than the complete virus consisting of the nucleoid plus outer membrane.

GRADES OF STRUCTURAL ORGANIZATION IN VIRUSES

It is possible, in principle, to classify viruses biochemically, according to the quantity of information coded in the nucleic acid and to the phase of cellular synthesis "captured" by the virus. However, many viruses contain non-genetic elements, i.e., constituents other than nucleic acid, that are, nevertheless, essential for their normal infective cycle. Thus, the myxoviruses possess protein components that have an attachment and enzymatic function necessary for the invasion of the cell. Such components may be normal cellular constituents, or closely related to them, and would be incorporated into the virus during the final maturation. For example, many viruses are encapsulated by a piece of cellular membrane as they are secreted from the cell. A complex virus of this kind might consist of a number of distinct parts, besides the primary nucleoprotein carrying the genetic information. Some very large and complex viruses, (such as vaccinia, which multiplies in the cytoplasm [Cairns, 1960]), may carry enzymes that have no normal cellular counterparts. The production of such viral enzymes would then have to be a concurrent part of viral multiplication. One could extend the range of organization to the elementary particles of PPLO (Morowitz et al.,

1962) which, though smaller than some viruses, are intact, free-living cells.

Indeed, this wide range of variation from simple viruses to the smallest living organisms is concrete evidence of a series of grades of structures of increasing complexity. This is an aspect of biological organization that has recently been discussed by Bernal (1959) in a rather different connection. The point emphasized by him is that the biological structures we observe are not arranged in a continuous order, but in a discontinuous one. Each type of structure seems to be composed of units of fairly definite sizes which come together to form a larger unit on the next level of organization.

When applied to infective agents, this concept would mean that the bare nucleic acids might be placed in the lowest grade. The next grade would contain the simple viruses in which the nucleic acid is now stabilized by protein molecules in the form of a protective shell. The next grade might involve the addition of a lipid coat. Higher grades would include the provision of specialized mechanisms for attachment to the cell and for penetration (these two functions are separate in bacteriophage, for instance). This is not to imply any hypothesis of stages in evolution from simple to more complex forms. Viruses undoubtedly mutate, and presumably evolve, but the direction of change may equally well be from a semi-autonomous form to a highly efficient simple form, as from a simple to a more complex form. In fact, since viruses could not possibly exist before cells, the minimal viruses could be considered a highly evolved form.

SUB-ASSEMBLY AND SELF-ASSEMBLY

The essential point about grades of organization is that large structures are built out of smaller structures. The components of a virus or a part of the living cell can be synthesized separately by a sub-assembly process (Crane, 1950) and then associated, following definite rules, to form a complete system. The advantages of such a process is that biological control can be exercised at each level of organization, so that even if mistakes can occur at the various stages, the defective components can be rejected. The net result is that very complex systems can be built up with high efficiency.

A production line is an apt analogy for some stages of the sub-assembly process used by a living cell. The synthesis of a polypeptide chain from amino acids is an example of such a process. However, once the peptide bonds of a protein are formed, the simple analogy of a production line breaks down. No template or other external direction appears to be needed to fold up many proteins; the stable configuration is evidently deter-

mined by the amino acid sequence and thus, ultimately, by the genetic code. Moreover, some proteins are capable of assembling themselves into highly organized structures. The assembly processes of a living cell are different in principle from those of a factory, in that the directions for constructing many complex biological structures are built into the constituent components. These biological structures are thus constructed by a self-assembly, and not merely a sub-assembly, process. One of the clearest examples of self-assembly in biology is provided by the simple viruses, in particular by tobacco mosaic virus, where the self-assembly process has been reproduced in vitro (Fraenkel-Conrat and Williams, 1955).

Self-assembly is a process akin to crystallization and is governed by the laws of statistical mechanics. A simple virus particle is distinct from a crystal in that it has a finite, well-defined size, and consists of two chemically and structurally very different components. The protein subunits and the nucleic acid chain spontaneously come together to form a simple virus particle because (under appropriate solvent conditions) this is their lowest energy state. It is in the transition from a state in which protein subunits and nucleic acid chains are randomly arranged in space to a state in which they are highly ordered that virus assembly is like crystallization. The driving energy for this process is provided by the formation of inter-subunit bonds. The order in the final structure is a necessary consequence of the statistical-mechanical compulsion to form the maximum number of the most stable bonds between the units. The molecules of a crystal are, in general, ordered in a completely regular way. Thus, in a crystal, all the molecules are in identical or at least physically indistinguishable environments. Arranging identical units in identical environments necessarily produces a symmetrical structure, and there are only a geometrically limited number of kinds of symmetry.

Any ordered structure, whether it is a crystal or a virus, will have some type of well-defined symmetry. However, as we have pointed out elsewhere (Caspar and Klug, 1963) an ordered structure built of complex molecules such as proteins, need not have all identical molecules in *exactly* identical environments. The important point is that the lowest energy structure will have the maximum number of most stable bonds formed—and this may be physically realized, as in icosahedral virus shells, by quasi-equivalent bonding of identical units. These physical considerations have led to an extension of traditional concepts of symmetry, more specifically applicable to highly organized biological structures.

Self-assembly requires built-in directions in the form of a structure unit with an inherent set of specific bond sites. A biologically significant feature of a self-assembly system is that it can be self-checking. Thus, if a mistake is occasionally made in the synthesis of a virus protein subunit, this defective unit is unlikely to have the required set of specific bond sites and would, therefore, not be incorporated into the virus package. The complete virus package requires a definite organization of protein and nucleic acid. As we will show, the structure of simple viruses is principally determined by the ordered packing of the protein subunits. However, mistakes in protein assembly may occur, but the interaction with the nucleic acid provides another checking mechanism at a higher level of organization that will tend to reject incorrectly assembled protein coats.

Of course, in the process of assembly of a complex system, it is generally necessary to have a controlled sequence for the production of the substructures. It often appears to be not enough, as with TMV, to have all the substructures made, and then wait for their chance aggregation. Thus, in the example of bacteriophage growth (see Kellenberger, 1961), the production of the tail protein units does not begin until the complete head is assembled, and, indeed, it appears that as soon as the subunits are formed, they are assembled into place at the base of the pre-formed head. Nevertheless, from a structural viewpoint, the protein subunits of the tail are potentially capable of self-assembly into a separate helical structure.

STRUCTURAL STUDIES

The recognition that a virus can be constructed by a sub- or even self-assembly process implies that the building rules by which it is constructed can be deduced from the properties of the finished product. The role of X-ray diffraction studies (see Klug and Caspar, 1960) has been to elucidate the way in which the components of simple viruses are arranged. From the detailed structural, chemical, and physico-chemical studies on TMV, a reasonable picture has emerged of the way in which this simple virus is constructed. The amount of experimental information available regarding the substructure of the simple isometric viruses is more limited than for TMV. However, on the basis of these results, and on the assumption that the construction of simple isometric viruses is governed by the same type of physical principles which apply to helical viruses, we have shown (Caspar and Klug, 1963) that there is only one kind of efficient design possible for their protein shells. Our deductions regarding the organization of simple viruses are also likely to apply to the primary

packaging of the nucleic acid of more complex viruses. Moreover, the same dynamic construction principles are likely to apply to organized cellular components, as well as to viruses. Thus, knowledge of the molecular architecture of viruses can also contribute to our understanding of the functional organization of parts of the living cell.

HELICAL VIRUSES

TOBACCO MOSAIC VIRUS

The structure of tobacco mosaic virus has been more intensively studied than that of any other virus and the results (see Fig. 1) have recently

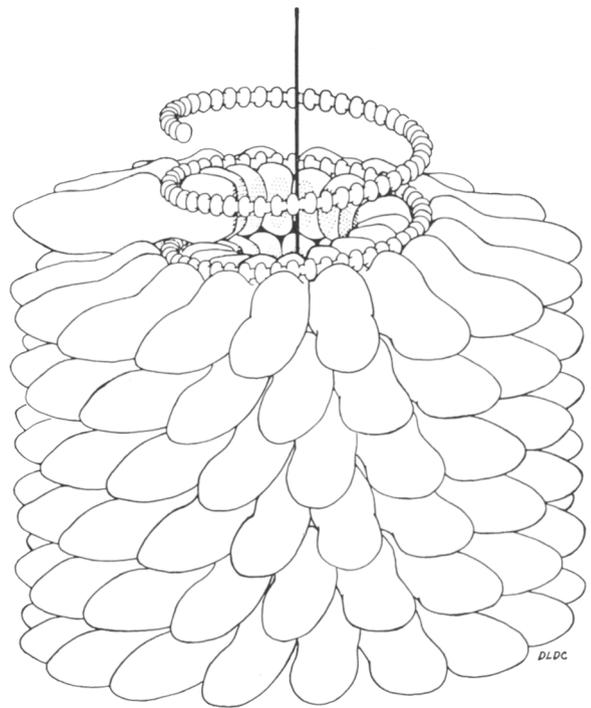


FIGURE 1. A drawing of a segment of tobacco mosaic virus (Klug and Caspar, 1960).

The shape of the protein subunits is rather schematic, and each nucleotide is represented by a flat disc. For clarity, part of the ribonucleic acid chain is shown without its supporting framework of protein but this regular configuration could not be maintained without the protein.

The diagram illustrates how each protein subunit is identically related to its neighbors and it will be clear how more subunits could be added regularly in a step-wise manner to build the virus particle.

been reviewed by us (Klug and Caspar, 1960). We have shown that, by combining the results from X-ray diffraction and other physical and chemical studies, it is possible to characterize the structure of the virus by a small set of accurately determined numbers.

The protein subunits of the virus are closely packed in helical array and any one subunit is

structurally, as well as chemically, indistinguishable from any other. The RNA chain is coiled in a compact way between the turns of the protein helix, and the phosphate-sugar backbone must be folded in such a way that successive groups of three nucleotides are equivalently related to each protein subunit.

The nature of the stability of the TMV structure has recently been summarized by Caspar (1960). The stability of an intact virus particle is much greater than that of the sum of its parts. Infectious RNA isolated from TMV is very labile, and the isolated protein subunits are easily denatured. Intact TMV, however, remains native and infectious over a period of decades at room temperature. This great stability is obviously the result of the interactions between the parts in the native virus structure.

The protein-protein interactions are the dominant ones, since the protein subunits can aggregate to form the same helical structure without the RNA. Hydrophobic bonds play a significant role in holding the subunits together, as may be deduced from kinetic studies of the polymerization-depolymerization reaction as a function of temperature and pH (Lauffer et al., 1958). The RNA of the virus has no intrinsic structure of its own, and its configuration in the virus is determined by the packing of the protein subunits. The RNA does, however, contribute significantly to the stability of the structure, since polymerized protein can be disaggregated under much milder conditions than either the native or reconstituted virus. Salt links presumably occur between the phosphate groups of RNA and basic groups of the protein. This cannot, however, be the only interaction. There is evidence (Fraenkel-Conrat and Singer, 1959) that the combination of TMV protein with its own RNA is more specific than with non-viral RNA or even that of distantly related strains. Such specificity might be accounted for by some regularity in at least part of the nucleotide sequence, since there are only three nucleotides associated with each subunit and the subunits are all indistinguishable. It has, therefore, been suggested (Caspar, 1960) that the RNA may contain the information necessary for it to link up with its own protein, as well as the "code" for the sequence of this protein.

At all events, the finished product—the intact TMV particle—is a remarkably stable object. The buried location of the RNA in the helical array of protein units accounts for its resistance to attack by ribonuclease. Its thermal stability in the virus, as compared to the lability of isolated RNA, is presumably due to its regular interaction with the protein.

The TMV particle is, thus, an exemplary piece

of intimate packaging of RNA by small protein molecules. Moreover—and this is most important—the protein subunits have the capacity to assemble themselves, with or without nucleic acid, to form the framework of the packaging. This property of self-assembly is fundamentally connected with the helical symmetry of the virus.

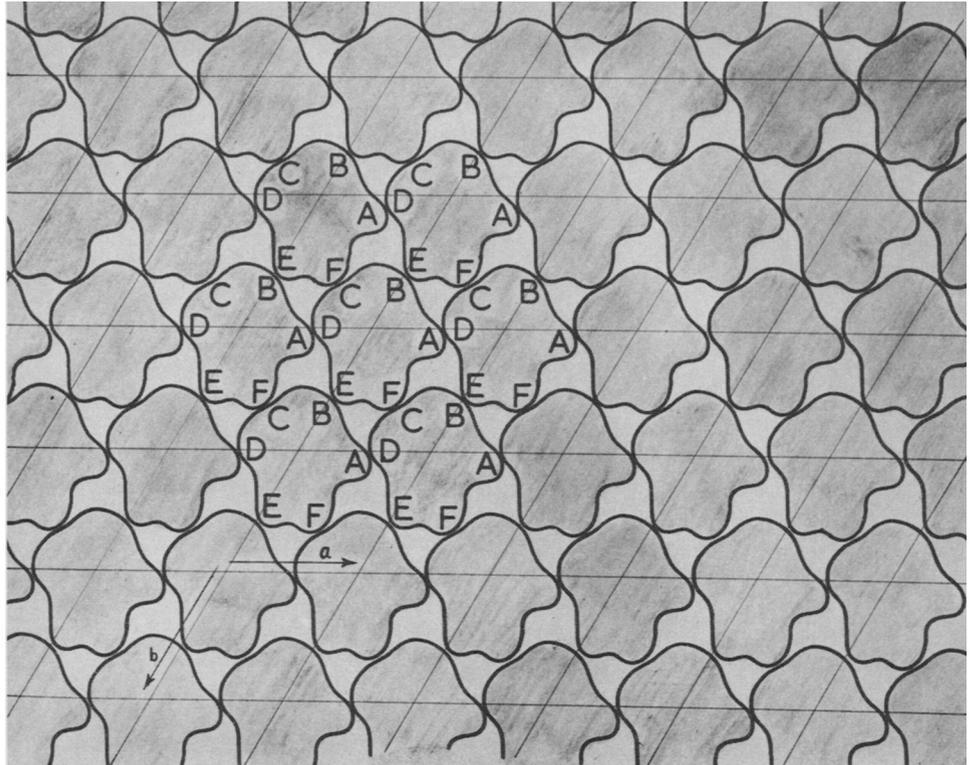
HELICAL SYMMETRY

The regular structure of the protein part of the virus particle is a result of packing identical units so that the same kinds of contacts are used over and over again. In the final structure, each subunit (except for those at the ends) is situated in the same environment, i.e., all subunits make the same bonds. They are, therefore, equivalent. This necessarily results in a symmetrical structure.

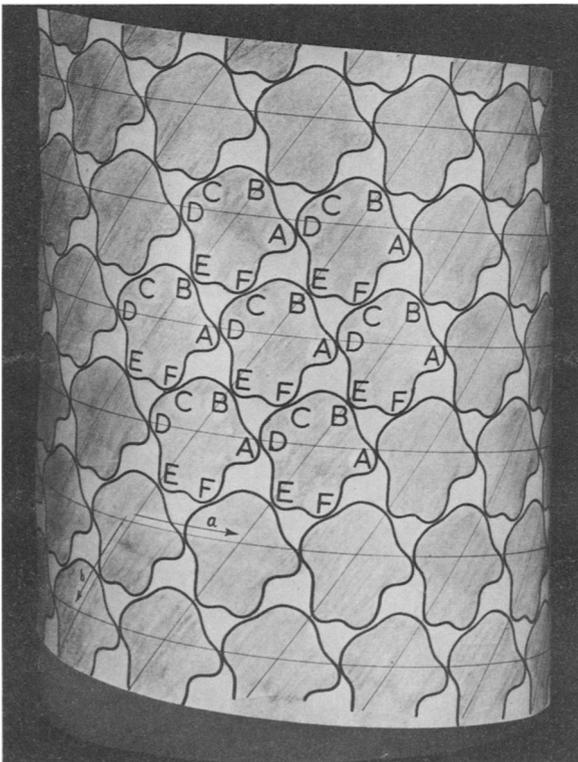
This argument may be reversed. A symmetrical structure can be divided into identical geometrical units (the asymmetric units) which are all equivalent. If, therefore, we wish to find all ways of packing units so that they are equivalently related, we can treat the problem abstractly by considering all the symmetries possible for the structure it is desired to build. This is essentially the mode of reasoning followed by Crick and Watson (1956) and by us in our theory of icosahedral virus structure.

The idea of equivalence may be illustrated in more concrete terms by the example in Fig. 2a, which shows a portion of a two-dimensional regular array of identical units, arranged in an approximately close-packed fashion. (The particular net chosen in the example is, in fact, the same as that which would occur on a cylindrical surface of radius equal to 60 Å picked out of the TMV structure.) It will be noted that, from a geometrical or, more strictly, topological point of view, each unit can be regarded as bonded to its neighbors by only three "bonds", namely AD, BE, and CF. Fixing these bond distances and the angles between them is enough to determine the structure. To realize these three "bonds", it is necessary to specify three "donor" and three "receptor" bonding sites on each unit. In fact, the minimum number of bonds for a lattice of this type is two, since the structure would still cohere if, say, the bond CF were eliminated.

A two-dimensional lattice of this kind (primitive lattice) can be rolled up into a cylindrical surface (of any diameter) without disturbing the bonding pattern geometrically (though, of course, with real units, physical considerations would enter). It is possible to roll up any plane lattice in such a way as to produce a helix (Fig. 2b). Although the number of helical symmetry types is limited (Klug, Crick and Wyckoff, 1958), the important point for



2a



2b

FIGURE 2. (a) Asymmetric units arrayed in a primitive plane net (i.e., a net possessing only translations, here a and b , and no rotational symmetry).

The arrangement has been chosen so that the units are close-packed. Each unit makes identical "bonds" with its neighbors. There are six bonding sites, A, B, C, D, E, and F on each unit, but note that there are only three different "bonds", namely, AD, BE, and CF.

(b) The net of Fig. 2(a) rolled up into a cylindrical surface to produce a helical array. Note that geometrical "bonding" pattern is undisturbed by the rolling, the same "bonds" still being made.

this paper is that in a helical structure there is no geometrical restriction on the helical parameters. The number of units per turn, for instance, need not even be integral.

PHYSICAL CONSIDERATIONS

The length of a helical structure, such as TMV, is not determined by its symmetry nor by its geometrical parameters, since the structure can repeat indefinitely in the direction of the helix axis. As will be obvious from Fig. 1, the additional feature necessary to determine the length of helical array of protein in the intact virus particle is the length of the RNA chain. An illustration of this idea may be found in the case of another helical virus, tobacco rattle, which has the same proportional content of RNA as TMV and a similar helical pitch, but is only about two-thirds the length (Harrison and Nixon, 1959; Nixon and Harrison, 1959). One would therefore expect that if the RNA is also in the form of a single chain determining the length of the virus, it would be situated at a radius in the particle about 3/2 times that in which it is located in TMV. Some preliminary X-ray studies (Finch and Klug, unpublished) indicate that this may indeed be the case.

It is not difficult to imagine how the virus is assembled, with the individual protein subunits coming together by a process akin to crystallization and enfolding, successively, ever increasing lengths of the RNA chain. The main difficulty, perhaps, is in envisaging how the process begins, and even this would be resolved if there were some specificity in part of the RNA chain for starting the growth of the protein helix. But, whatever the detailed mechanism, the process can be carried out without the mediation of any organizing principle, as the classical experiment by Fraenkel-Conrat and Williams (1955) of reconstitution *in vitro* demonstrates.

FLEXIBLE RODS

There are now many examples known of flexible filamentous viruses, particularly among the plant viruses (see Horne, Russell, and Trim, 1959; Klug and Caspar, 1960). The most notable instances, in the case of animal viruses, are the flexible nucleoprotein filaments occurring in the interior of the myxoviruses (Horne et al., 1960). These sinuous structures may be classed together with the rigid, rod-shaped viruses, since they are undoubtedly helical, but with the difference that they are not held rigid by strong interactions between the successive turns of the helix. In the example of Fig. 2b, for instance, it is as if the bonds AD along the helix remained strong but, by comparison, the strengths of the bonds BE and CF were greatly

reduced, or perhaps even more likely, the bonding sites, B, C, E, and F were made more non-specific.

Strictly speaking, the subunits in a sinuous helical virus can no longer be exactly equivalent, since this would demand a straight axis of symmetry. However, since the local bonding pattern would not be changed very much when the helix axis is slightly bent, the subunits in a flexible helical structure can, nevertheless, remain *quasi-equivalently* related.

There is no long-range regularity in the departures from equivalence in a randomly-flexed helix. However, even in a highly ordered structure, identical units need not be packed in exactly equivalent environments. The X-ray studies of Caspar and Holmes (1963) on the dahlemense strain of TMV indicate that there is a periodic perturbation in the packing of the subunits near the outside surface of the virus which leads to a small, regular deformation of the helix in the axial direction. The outer part of the protein subunit can bend into slightly different positions so that chemically identical parts of different molecules are packed in quasi-equivalent environments. The energy for this deformation comes from a weak interaction between the outer parts of the subunit.

The observations of Mattern (1962), discussed below, suggest that the normal helical structure of TMV may also be regularly perturbed under some conditions of specimen preparation for electron microscopy.

ICOSAHEDRAL VIRUSES

Most of the experimental information available on regular substructure in isometric viruses is summarized in two recent reviews, the one principally concerned with X-ray diffraction results (Klug and Caspar, 1960) and the other with electron microscope observations (Horne and Wildy, 1961). Much of the experimental work on "spherical" viruses has been done since the hypotheses of Crick and Watson (1956; 1957) were put forward.

CUBIC SYMMETRY

We have already discussed, in connection with helical structures, how the use of the same contact points over and over again in packing subunits necessarily leads to a symmetrical structure. Crick and Watson (1956) realized that, out of all the types of symmetry possible for a structure of limited extent (called the point-groups—these have long been enumerated mathematically), only the cubic point groups were likely to lead to an isometric particle. The essential point about cubic symmetry is that the three coordinate directions in space are not independent; in fact, they are equivalent, so

that no direction in space can be preferred. Three types of cubic symmetry exist; namely, tetrahedral (2:3), octahedral (4:3:2) and icosahedral (5:3:2). For a virus particle these imply, respectively, 12, 24, or 60 identical subunits, arranged identically on the surface of a sphere. Models in which ping-pong balls (Caspar, 1957; Klug et al., 1957) are used to represent the subunits do not bring out the point that the asymmetric unit can be of any shape and be provided with more or less specific bond sites. This is best understood by looking at a model made with units having no symmetry, as in Fig. 3, which demonstrates the structural equivalence in the case of icosahedral symmetry.

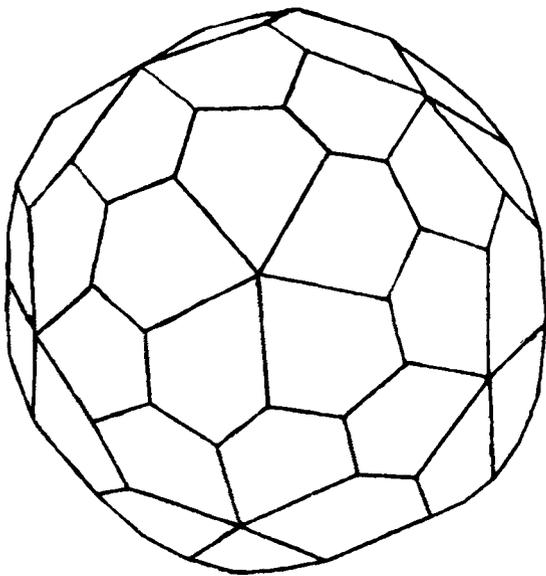


FIGURE 3. A diagram to illustrate the meaning of strict equivalence. The 60 identical polygonal units are arranged according to icosahedral symmetry.

An edge between two adjacent polygons may be regarded as the contact between units. Each unit makes identical contacts with its neighbors.

ICOSAHEDRAL SYMMETRY

Crick and Watson made no attempt to assess the relative merits of the three types of cubic symmetry. Since that time, there has accumulated a large body of evidence that the icosahedral symmetry is preferred in virus structure. Indeed, no well-established examples exist at present of isometric viruses which are not icosahedral (the reported cases of octahedral bacteriophages are dealt with below). The first experimental evidence for icosahedral symmetry in a virus came from the X-ray diffraction studies of Caspar (1956) on tomato bushy stunt virus, soon followed by that of Klug, Finch, and Franklin (1957) on turnip yellow mosaic virus. These investigations confirmed the

prediction of Crick and Watson that these viruses possess cubic symmetry and are, therefore, built out of subunits; moreover, they also showed that this symmetry was icosahedral.

Shortly thereafter, electron microscope observations showed that a number of viruses had the shape of a regular icosahedron—the most conclusive instance being that of *Tipula* iridescent virus (Williams and Smith, 1958). The fact that the external shape is icosahedral does not necessarily mean that the symmetry down to the molecular level is also icosahedral. Thus, for example, various Radiolarians (D'Arcy Thompson, 1952) with highly symmetrical skeletons are probably not built of a regular array of silica units.

When an X-ray diffraction investigation of poliovirus (Finch and Klug, 1959; see Fig. 4) also revealed icosahedral symmetry, it seemed fairly certain that the occurrence of icosahedral features in quite unrelated viruses was not a matter of chance selection. These results, on poliovirus, led to the conclusion that there are no structural grounds for distinguishing the smaller animal from the plant viruses. Moreover, the question was raised (Finch and Klug, 1959) whether there was not another general principle at work here, to be added to those already put forward by Crick and Watson (1956, 1957). The full answer could not then be given, but the point was made that the advantage of icosahedral symmetry over the other types was that it allows the use of the greatest possible number, namely 60, of identical asymmetric units to build a spherical framework in which they are also identically packed. If, therefore, it is desired to build a shell of a given size as economically as possible, i.e., with the smallest subunits feasible, icosahedral symmetry would naturally be preferred.

It was also pointed out that if one desired to "enclose" a space around a central point by a set of domains on a closed surface, the ratio of the number of domains to the surface area covered is smallest if icosahedral symmetry is employed. (This may be shown by adapting some of the geometrical theorems to be found in, say, the book by Toth [1953]). It, therefore, seemed likely that icosahedral symmetry would also be the most efficient form of packing, but these notions were left quite imprecise, until very recently, when we formulated what we believe to be a satisfactory theory (Caspar and Klug, 1962) for the efficient design of closed shells, which demands the use of icosahedral symmetry. It will be seen that the rather vague ideas of economy and efficiency are not necessary, and that both are merely aspects of the more comprehensive idea of the optimum design of a shell.

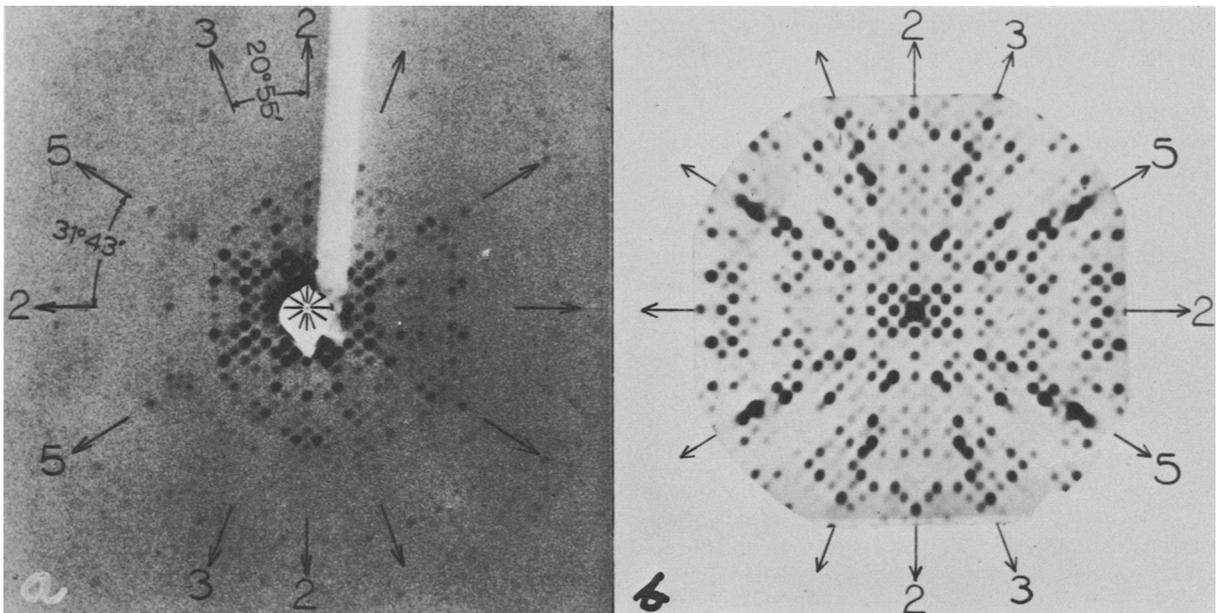


FIGURE 4.

(a) An X-ray diffraction pattern of a poliovirus crystal (Finch and Klug, 1959). There are spikes of high intensity along certain directions which are related as the 5- and 3- and 2-fold axes of an icosahedron (as indicated by the arrows.)

(b) An optical diffraction pattern of 60 points on the surface of a sphere with icosahedral symmetry. The intensity distribution of the poliovirus pattern shows the same symmetry relations as this optical analogue.

EXPERIMENTAL BACKGROUND

The theory stems, essentially, from three different sets of experimental observations.

(1) *X-ray diffraction results.* The X-ray diffraction photographs taken so far of viruses mostly relate to fairly large or moderate spacings in the virus particle. In a number of cases, photographs have been obtained extending to spacings as low as 5 Å (turnip yellow mosaic virus [Klug and Finch, 1960]; tomato bushy stunt virus [Caspar, Finch, and Klug, unpublished]). These show that icosahedral symmetry is present down to the molecular level in a substantial proportion of the particle. It cannot be proved that the whole of the virus particle has strict icosahedral symmetry—indeed, this would be most unlikely in view of the presence of a long molecule of nucleic acid—but it is likely that most of it has. These questions of the occurrence of pseudo, partial, or merely statistical symmetry are rather complicated ones, and are further discussed in the papers by Klug and Finch (1960), and Klug and Caspar (1960). If the protein shell has strict icosahedral symmetry, then it must be built up of either 60 subunits, or a multiple of 60. We shall call these *structure units*.

(2) *Chemical evidence.* In the cases where the problem has been investigated, the chemical subunits have been found to be all identical and, moreover, of molecular weight about 20,000, (turnip yellow mosaic virus: Harris and Hindley

[1961]; tobacco mosaic virus: Harris and Knight [1955]; wild cucumber mosaic virus: Yamazaki and Kaesberg [1961]).

In other cases, where the chemical evidence is not complete, estimates of minimal molecular weight from the amino acid composition, or from degradative studies, suggest that it is unlikely that the units have a molecular weight greater than about 50,000. We have already referred to the theoretical considerations (cf., Crane, 1950; Crick and Watson, 1957) which suggest that the size of protein molecules is limited.

(3) *Electron microscope observations.* As soon as the first high resolution electron micrographs of an icosahedral virus were obtained, beginning with adenovirus (Horne et al., 1959) and turnip yellow mosaic virus (Huxley and Zubay, 1960; Nixon and Gibbs, 1960), it seemed that there was a structural paradox. The number of morphological units observed, so far, on the surface of icosahedral viruses is never 60 or a multiple of 60, and in most cases it is greater than 60. A full account may be found in the review by Horne and Wildy (1961), and the subject will be dealt with by Wildy at this symposium.

We have already discussed the resolution of the paradox in terms of relationship between symmetry and morphology at some length (Klug and Caspar, 1960; Klug and Finch, 1960), and have attempted to draw the distinction between the various kinds

of unit that will be revealed by different techniques. We have preferred to use the term "morphological units" for the surface features revealed by electron microscopy, rather than "capsomeres" (Lwoff, Anderson, and Jacob, 1959) since this does not prejudice the question as to whether these units are the actual *building* units of the shell, or whether they merely represent rather close clusters of structure units. The theory now to be presented shows that the appearance of "capsomeres" may merely be an aspect of the mode of assembly of structure units.

THE GEOMETRY OF ICOSAHEDRAL VIRUSES

THE PROBLEM

If we accept the value of 20,000 as a working figure for the molecular weight of a chemical subunit, we may ask how many are needed for the formation of spherical shells of different sizes. Taking the shape of such a unit as an ellipsoid of $80 \times 25 \times 25 \text{ \AA}$ (on the analogy of tobacco mosaic virus), or a sphere of diameter 36 \AA , 60 such units more or less close-packed in accordance with icosahedral symmetry would lead to a spherical shell of outer diameter about 150 \AA to 200 \AA . This is about the diameter of the smallest known viruses (Loeb and Zinder, 1961; Kassanis and Nixon, 1960), and of ferritin (Harrison, 1959) which, being a packet of colloidal iron in a protein shell, we believe to be constructed on the same principle.

The next largest diameter commonly found in the small viruses so far studied is about 280 \AA . This would require from about 150–250 of the above hypothetical subunits, so that a multiple of 60 is required if icosahedral symmetry is to be used. In the case of turnip yellow mosaic, and wild cucumber mosaic virus, it has been established by chemical analysis that the protein shell is built up of more than 60 identical molecules.

Now, it is impossible to put more than 60 identical units on the surface of a sphere in such a way that each is identically situated. Indeed, as stated above, the only such numbers possible are 12, 24, and 60. If $60n$ units are put on the surface of a sphere, the best that could be done is to arrange them in sets of 60 units each, but the members of different sets cannot be equivalently related. If the structure were built out of n different types of unit, there would be no conceptual physical difficulties and, indeed, no problem. However, the purpose of the theory is to see how far we can go with all units identical. Moreover, we do not wish to drop the essentially physical principle at the basis of Crick and Watson's reasoning that, in the formation of the shell, the same contacts between

subunits are used over and over again, since it is this principle which, we believe, is the key to the organization of the units in a regular virus. Thus stated, there is only one way out of the dilemma. We must drop the insistence on strict mathematical equivalence, but retain its physical essentials. This could be done if we could find a method of arranging more than 60 units on the surface of a sphere so that they are quasi-equivalently related.

QUASI-EQUIVALENCE

The solution we have found (Caspar and Klug, 1963) was, in fact, inspired by the geometrical principles applied by Buckminster Fuller in the construction of geodesic domes (for an account, see Marks, 1960). The resemblance of the designs of geodesic domes (e.g., Fig. 5) to icosahedral viruses had attracted our attention at the time of the poliovirus work (Klug and Finch, 1959, quoted in Marks, 1960, p. 44). Fuller has pioneered in the development of a physically orientated geometry based on the principles of efficient design. Considering the structure of the virus shells in terms of these principles, we have found that with plausible assumptions on the degree of quasi-equivalence required, there is only one general way in which iso-dimensional shells may be constructed from a large number of identical protein subunits, and this necessarily leads to icosahedral symmetry. Moreover, virus subunits organized on this scheme would have the property of self-assembly into a shell of definite size.

The basic assumption is that shell is held together by the same type of bonds throughout, but that these bonds may be deformed in slightly different ways in the different, non-symmetry related environments. Molecular structures are not built to conform to exact mathematical concepts but, rather, to satisfy the condition that the system be in a minimum energy configuration. We have seen above how, in the dahlemense strain of TMV (Caspar and Holmes, 1963), a small departure from equivalent packing of identical units may result in order to achieve this condition.

It is important to consider what degree of deformation may be tolerated in the packing of the protein subunits. We may take, as a rough guide, the deductions of Pauling (1953) from a study of the interactions between antigens and antibodies. He has concluded that "the combining regions may be about 10 \AA in diameter, and that the amount of leeway in juxtaposition of atoms may amount to about 0.5 \AA . This amount of leeway corresponds to a flexibility of the bond, permitting bending by 5° in any direction from the average bond direction." The maximum degree of non-equivalence required between subunits in our models for icosahedral

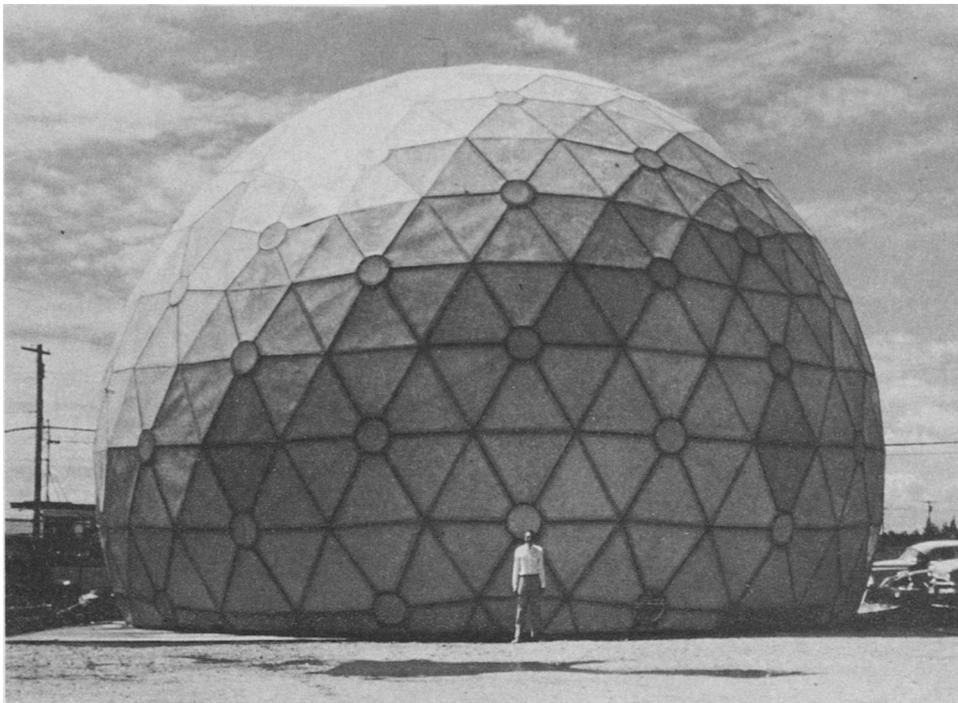


FIGURE 5. A Fuller geodesic dome (Radome designed by Geometrics, Inc., Cambridge, Mass.). Note that the surface is made up of quasi-equivalent triangles and that these are grouped in hexamers and pentamers about the small rings of the dome. (Photograph supplied by W. H. Wainwright).

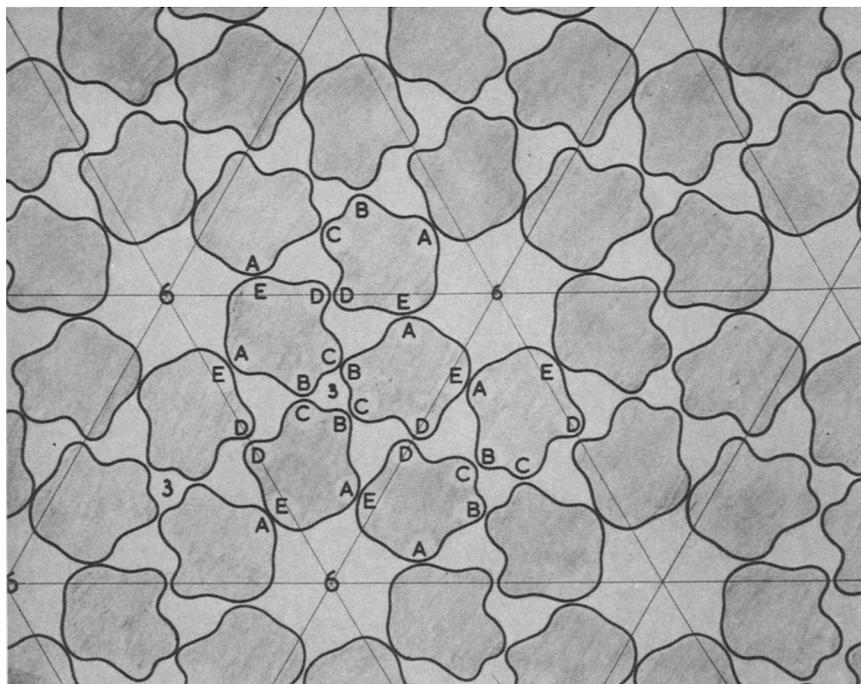


FIGURE 6. Asymmetric units arrayed in an equilateral-triangular plane net. Besides having translations, here a and a , the lattice has 6-fold rotational axes of symmetry. Although the asymmetric units are in 6 different orientations in space, they are all exactly equivalently related.

Each unit here is equipped with five "bond" sites, A, B, C, D, and E, forming three different "bonds", namely a hexamer bond AE, a trimer bond BC, and a dimer bond DD. (Note that only two of these bonds are absolutely essential for coherence of the array.)

shells is of this order, so that we may quite properly refer to the units as quasi-equivalent.

The meaning of quasi-equivalence can be illustrated as follows. In the omni-triangulated geodesic dome shown in Fig. 5, the complete sphere would consist of 720 triangular units, but although they are actually of 12 different types, they are all very similar. The asymmetric unit of this radome consists of these 12 symmetrically distinct triangles, but the physical subunit may be considered as one "average" triangle.

FOLDING OF PLANE NETS

The example just described shows that a quasi-equivalent bonding pattern between subunits can be obtained, even when more than 60 units are used. The device of triangulating the sphere into as equal subdivision as possible obviously provides the basis for the derivation of other geometrically

quasi-equivalent packings on the sphere. The way to enumerate all the possible quasi-equivalent subdivisions systematically is to consider the triangulations of the sphere as derived from the folding up of a plane equi-triangulated net into a polyhedron with icosahedral symmetry. The justification of this procedure is given in the detailed paper by Caspar and Klug (1963) where it is shown that there is no other way of achieving a comparable degree of quasi-equivalence. The reasons for this may be understood from the diagrams in Figs. 2 and 7 which illustrate the folding of plane nets.

Consider the plane net in Fig. 2a, in which the units are related only by translations. This may be folded into a cylindrical surface (as in Fig. 2b) by cutting it along two parallel lines which join lattice points, and joining the two lines together.

The folding of a plane surface into a portion of a *doubly-curved* surface, may be illustrated by the

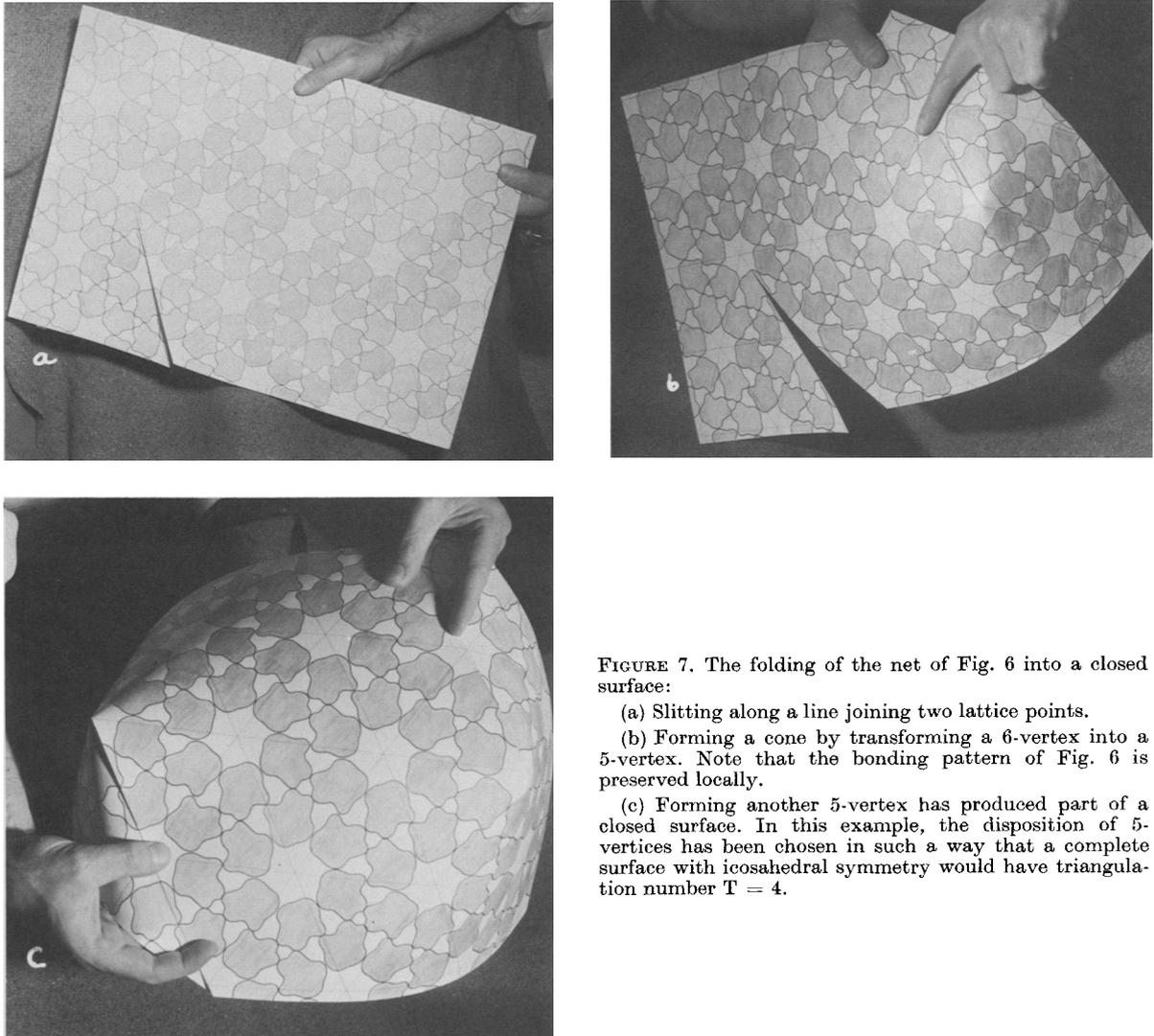


FIGURE 7. The folding of the net of Fig. 6 into a closed surface:

- (a) Slitting along a line joining two lattice points.
- (b) Forming a cone by transforming a 6-vertex into a 5-vertex. Note that the bonding pattern of Fig. 6 is preserved locally.
- (c) Forming another 5-vertex has produced part of a closed surface. In this example, the disposition of 5-vertices has been chosen in such a way that a complete surface with icosahedral symmetry would have triangulation number $T = 4$.

process of forming the sides of a cone or pyramid from a sheet of paper (Fig. 7). It is necessary to slit the paper along two lines, which are not parallel to each other, and join these together so that their point of intersection becomes the vertex of the cone or pyramid. This cannot be done with the plane net of Fig. 2a, without destroying the bonding pattern in the lattice.

In fact, it can be shown (Pawley, 1962) that there are only two types of plane nets (without mirror symmetry) which can be folded onto the surface of convex polyhedra and still maintain the same nearest neighbor contact pattern (cf., Figs. 6 and 7). They are the two plane lattices which have four-fold or six-fold rotational symmetry and which are, therefore, based on the square and equilateral triangular nets, respectively. A square net can only be folded into a cube, so that at the corners of the cube, only three edges meet, where four had met in the plane. If with each lattice point we have to associate a real unit, having extension above and below the infinitesimally thin theoretical surface, the strain will obviously be much larger than the tolerances we have allowed for quasi-equivalence.

A triangular net (Fig. 6) can be folded into a convex surface if 5, 4, or 3 of the triangular facets join at a polyhedron vertex, instead of 6, as in the plane. If only polyhedral vertices of one kind are introduced to form the closed surface, then it is easy to show topologically (cf., D'Arcy Thompson, 1952, pp. 732-740) that either twelve 5-vertices, six 4-vertices, or four 3-vertices are required. If these vertices are disposed symmetrically—there is no topological requirement that they be—then regular polyhedra will be formed, namely the icosahedron (all 5-vertices), the octahedron (all 4-vertices) or the tetrahedron (all 3-vertices). When real subunits are associated with the plane net, the departure from equivalence will obviously be smallest when a vertex of the plane net (a 6-vertex) is transformed into a 5-vertex (Fig. 7). For optimum design, therefore (i.e., to achieve the greatest degree of quasi-equivalence) it is clear that, of the three possible polyhedra, the icosahedron is to be preferred, by far.

DELTAHEDRA

A polyhedron whose faces are all equilateral triangles is called a deltahedron. Deltahedra models can be constructed from folded cardboard nets of equilateral triangles (Fig. 8). We have enumerated all possible deltahedra which have icosahedral symmetry ("icosadeltahedra") (Caspar and Klug, 1963). The icosahedron itself has 20 equilateral triangular faces, and any icosadeltahedron has $20T$ facets, where T is the *triangulation number* given by the rule: $T = Pf^2$ where P can be any number of the series 1, 3, 7, 13, 19, 21, 31, 37 ($= h^2 + hk + k^2$, for all pairs of integers h and k having no common factor) and f is any integer. For a fixed value of P , increases in f from 1 upward

correspond to successive subtriangulations of the primitive deltahedron. The deltahedra of the class $P = 1$ (Figs. 8a and b) can, therefore, be considered as higher orders of the icosahedron, and those of class $P = 3$ (Figs. 8c and d) as constructed from a pentagonal dodecahedron with pentagonal pyramids placed on its faces. These two classes are the only deltahedra which have planes of symmetry. All deltahedra for which $P \geq 7$ are skew, as is evident from Figs. 8e-h. These deltahedra are, therefore, enantiomorphous, existing in right- and left-handed forms.

The structure unit implicitly associated with the cardboard models can be visualized as one-third of a triangular face, so that the number of (quasi-equivalent) subunits = $60T$. Models for icosahedral virus shells can be constructed using a deltahedron core. Two such models are shown in Figures 9 and 10 of shells with triangulation numbers $T = 3$ and $T = 4$. In Figs. 9a and 10a, the positions of the structure units are represented by $60T$ wooden pegs, that is, three to each deltahedron face. Protein molecules are represented by pieces of rubber tubing. These structure units have been clustered in hexamers and pentamers, giving 32 morphological subunits in the model shown in Fig. 9b, and 42 morphological subunits in Fig. 10b.

The particular packing arrangements used in the figures has been chosen to give an appearance similar to that of electron micrographs of viruses. The important point about these models, however, is that the same type of contacts are used between all the structure units in the shell, even though they are not all in equivalent environments. That is why, in the wooden peg models of Figs. 9a and 10a, one cannot place a wooden peg in what appear to be gaps in the arrangements—any protein subunit placed at such a point would have to be quite differently bonded from the others.

MORPHOLOGICAL UNITS AND ICOSAHEDRAL CLASSES

The arrangement of structure units into rings of 5 and 6 is a geometrical necessity (Fig. 7), but the clustering into pentamers and hexamers is not. Clustering, however, provides a way of maximizing the contacts between the structure units at the outside surface of the shell. Unless the units are wedge-shaped, there must be increasing gaps between them at increasing distance outward from the surface of closest packing. Clustering into hexamers and pentamers produces morphological units arranged in a close-packed array on the surface of the shell. In this way, each hexamer has six nearest neighbors and each pentamer has five nearest neighbors. This close packing is a necessary consequence of the clustering about the vertices of the plane triangular net.

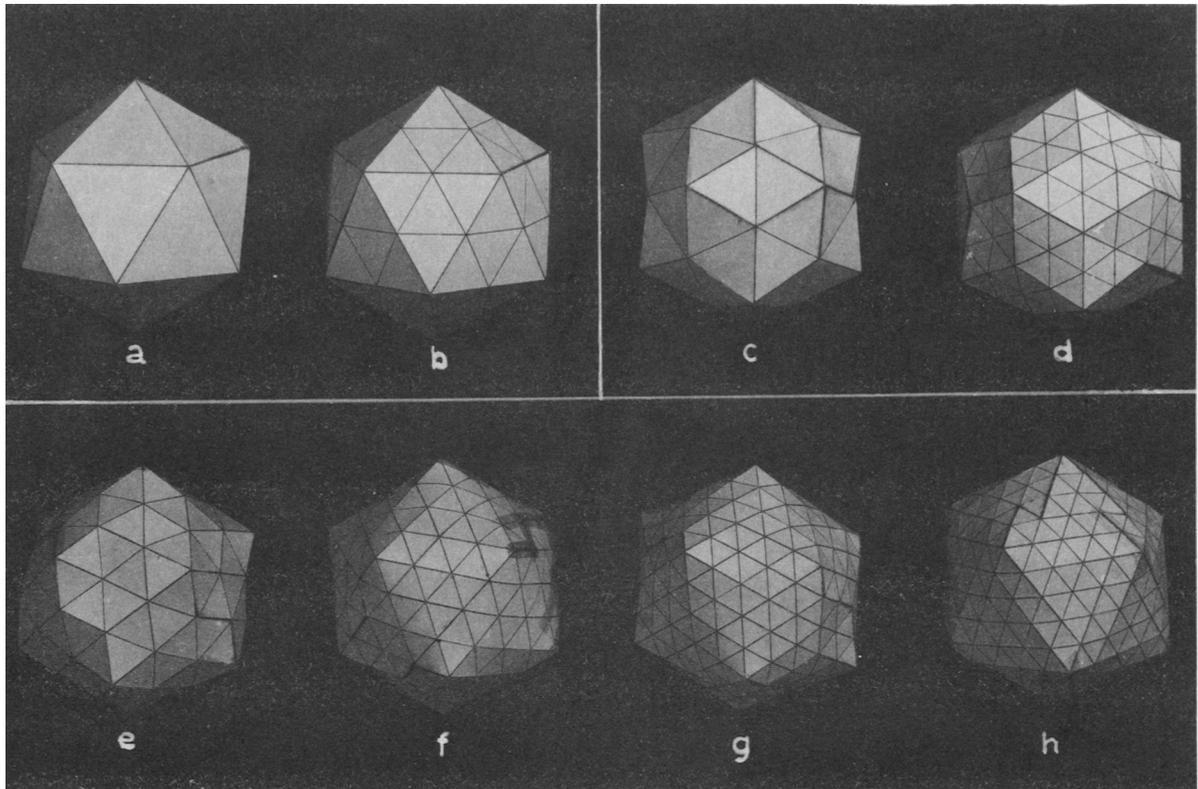


FIGURE 8. Deltahedra with icosahedral symmetry ("icosadeltahedra"). Each deltahedron has $20T$ equilateral triangles on its surface. T is called the triangulation number.

(a) and (b) are the two lowest members of the class $P = 1$ (triangulation numbers 1 and 4, respectively).

(c) and (d) are the two lowest members of the second class $P = 3$ ($T = 3$ and 12, respectively).

(e), (f), (g) and (h), first members of the skew classes $P = 7, 13, 19,$ and $21,$ respectively. The skewness can be seen from the orientation of the small triangles relative to the large triangle corresponding to an icosahedron face formed by connecting three neighboring 5-fold vertices.

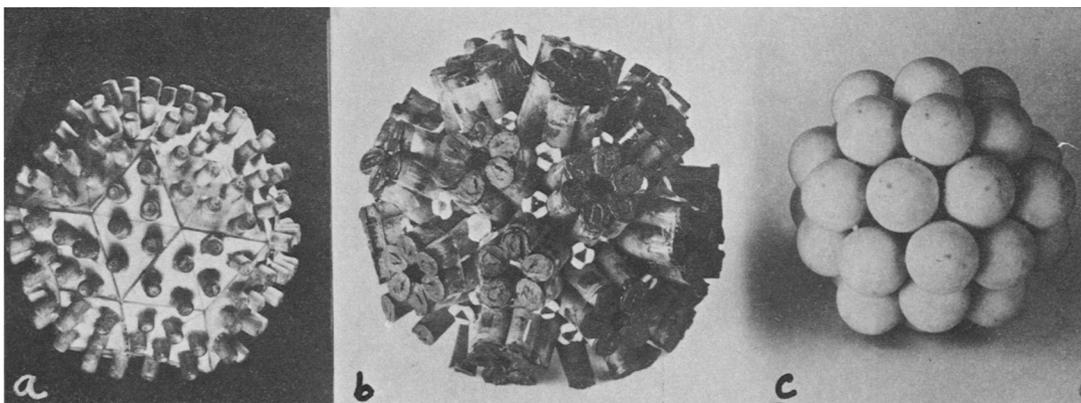


FIGURE 9. Models representing the arrangement of subunits in the icosahedral shell $T = 3$.

(a) Each subunit is represented by a wooden peg. There are 3 subunits per face of the underlying deltahedron (Fig. 8c), making 180 in all.

The particular disposition of the 3 subunits in a deltahedron face would be determined by the bonds involved, but the 3 subunits are always related by a local 3-fold axis (See Fig. 10a).

(b) Each subunit is now represented by a piece of rubber tubing in order to indicate its radial extent. The subunits are clustered at the outer surface into 20 hexamers and 12 pentamers to give 32 morphological units. (Other types of clustering are in principle possible—see text).

For a more realistic model of the same structure in which the bonding between subunits is represented, as well as their arrangement, see Fig. 12.

(c) The 32 morphological units in (b) are now all merely represented by ping-pong balls, in order to emphasize the appearance of the outer surface as seen at lower resolution. The model resembles the electron micrographs of turnip yellow mosaic virus (Huxley and Zubay, 1960; Nixon and Gibbs, 1960).

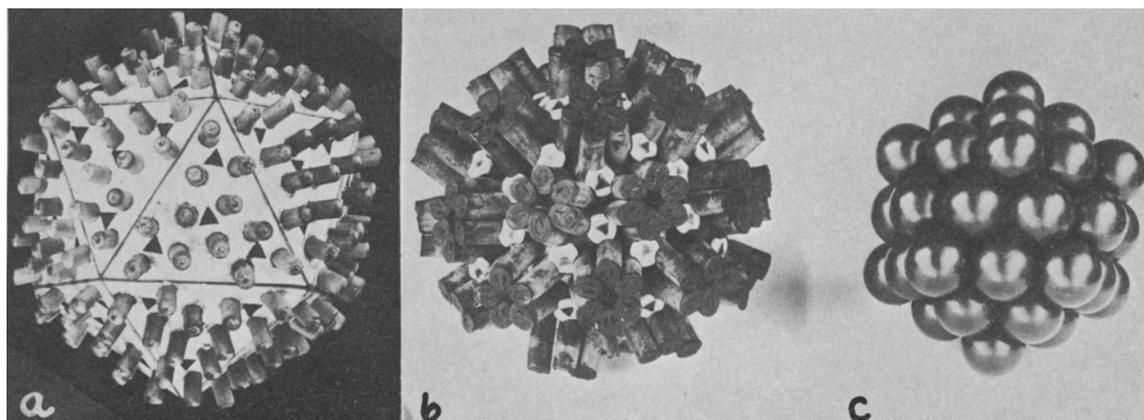


FIGURE 10. Models representing the arrangement of subunits in the icosahedral shell $T = 4$ (see Fig. 9 for details).
 (a) Each subunit is represented by a wooden peg. There are 3 per face of the underlying deltahedron (Fig. 8b), making 240 in all. The local 3-fold axes relating the 3 subunits per deltahedron face are shown by the black triangles.
 (b) Subunits represented by pieces of rubber tubing and clustered into 30 hexamers and 12 pentamers to give 42 morphological units.
 (c) Appearance of (b) at low resolution.

Clustering into 20 T trimers, 30 T dimers, or separation into 60 T monomers is also possible. With trimer clustering, each morphological subunit would have three nearest neighbors, rather than the five or six nearest neighbors for pentamer-hexamer clustering. The electron micrographs of poliovirus (Horne and Nagington, 1959) show some indication that each morphological subunit has only three nearest neighbors, thus these morphological units might be trimers of structure units.

It is a simple matter to calculate the number of morphological units that would be produced by a clustering of the subunits into hexamers and pentamers. There are $10(T - 1)$ hexamers plus 12, and only 12, pentamers. Values for the different classes are listed in Table 1. Our derivation (Caspar and Klug, 1962) of the possible numbers of morphological groupings is, necessarily, complete. The equations given by Horne and Wildy (1961, Table 4) are incomplete, despite redundancies. The reason for this is that their considerations are

based on empirical rules which are unrelated to the essential geometrical principles involved in icosahedral shell design.

So far, all icosahedral viruses, whose surface structures have been established, fall into the two classes, $P = 1$ and $P = 3$ (Fig. 11). Horne and Wildy (1961) noted this fact and suggested that they have some selective advantages over the skew families on the grounds that they are "relatively unstrained". However, it is not meaningful, in geometric terms, to say that the skew classes are more "strained" than the two non-skew ones.

The skew classes do have two structural properties which might make their use by nature unlikely. First, they can be built in a left- and right-handed form, using the same structure unit. One "hand" might be selected by the nucleic acid, but there would still be the chance that mistakes in assembly leading to defective particles might occur frequently. The second point follows from the dynamic aspects of icosahedral shell construction,

TABLE 1. THE CLASSES OF ICOSAHEDRAL DELTAHEDRA (SEE ALSO FIG. 8)
 Tabulation of the Triangulation Number T

Class								
$P = 1$	1	4	9	16		25	27
$P = 3$		3		12			
Skew Classes			7	13		19	21
Triangulation No. $T = Pf^2$	where $P = h^2 + hk + k^2$, h and k any pair of integers with no common factor and $f = 1, 2, 3, 4, \dots$							
No. of structure units $S = 60T$								
No. of morphological units $M = 10T + 2$								
	$= 10(T - 1)$ hexamers + 12 pentamers							

Some established virus examples (for references, see text)
 Phage ϕX , $T = 1$; Turnip yellow mosaic virus $T = 3$; Herpes, Varicella $T = 16$;
 Adenovirus, Infectious canine hepatitis $T = 25$.

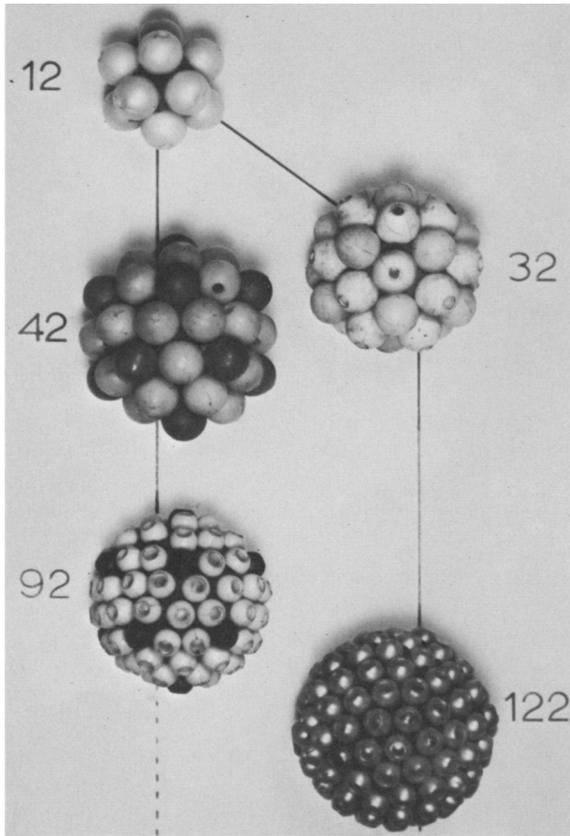


FIGURE 11. The arrangement of hexamer and pentamer morphological units in the lower members of the two icosahedral classes $P = 1$ (at left) and $P = 3$ (at right). The units are necessarily in close-packed array on the surface. The numbers of morphological units in the two classes are:
 $P = 1$. 12, 42, 92, 162, 252 See Table 1
 $P = 3$. 32, 122, 272
 In some of the models, the 5-coordinated and 6-coordinated units are shown in different shades.

which are discussed by Caspar and Klug (1963). Since the range of differences of the quasi-equivalent environments are greater for the skew classes than for the classes $P = 1$ and 3, there will be a corresponding difference in their respective distortion energies.

It is difficult to assess, theoretically, the relative advantages of the classes $P = 1$ and $P = 3$ for shell building. In the class $P = 1$, the range of the differences in the quasi-equivalent environments is less than that of $P = 3$, so that the deformation energy is correspondingly less. It is not surprising, therefore, that most of the viruses studied fall in the class $P = 1$. However, there is no difficulty in efficiently constructing the class $P = 3$. The lowest member of the class $P = 3$, $f = 1$, with 32 morphological units, has been established in the case of turnip yellow mosaic virus (Huxley and Zubay, 1960; Nixon and Gibbs, 1960) and is also

believed to be the arrangement followed in an ECBO virus (Fowle et al., 1962). No higher members of this class have so far been observed, and it will be interesting to see whether any exist.

COMMENTS ON INTERPRETATIONS OF SOME ELECTRON MICROSCOPE OBSERVATIONS

(a) Many studies with the electron microscope have revealed an array of morphological units on the surface of various viruses. Most of the units seen are surrounded by six neighbors (i.e., 6-coordinated) but sometimes a unit is seen which is 5-coordinated. It should be mentioned that if at least one 5-coordinated morphological unit is seen, and if the particle is isometric, then it is highly likely that the arrangement of morphological units is icosahedral. But it should, at the same time, be emphasized that an icosahedral arrangement cannot be regarded as established until: (1) at least two neighboring 5-coordinated morphological units can be unequivocally identified, and (2) the arrangement of 6-coordinated morphological units in their neighborhood can be discerned. This condition implies that the total number of morphological units can also be deduced without any ambiguity. Examples in which we would regard the number as established are adenovirus (Horne et al., 1959), turnip yellow mosaic virus (Huxley and Zubay, 1960; Nixon and Gibbs, 1960), Herpes virus (Wildy et al., 1960), infectious canine hepatitis virus (Davies et al., 1961), and Varicella virus (Almeida et al., 1962).

(b) In this connection, we might mention some cases where we believe the "counting" to be uncertain. It has been reported that both polyoma virus (Wildy et al., 1960) and human wart virus (M. G. Williams et al., 1961) consist of 42 morphological units. The published electron micrographs do not seem to us to establish these claims. Many of the virus particles show more than 30 odd knobs on the surface. If only half (or probably less) of the surface is revealed by the technique, the total number must be at least seventy. Moreover, the number of knobs seen around the periphery is about 16 or 17, which, again, is not consistent with a total of 42. Although some five-coordinated units can be observed, there are no clear cases where a pair of five-coordinated units are seen on either side of a single six-coordinated unit, as would be expected if the number of morphological units is 42. Mattern (personal communication) has also questioned the validity of the reported "counts" for these viruses.

Polyoma, human wart, and Shope papilloma virus particles all have similar morphology and

may each have the same number of morphological units. R. C. Williams et al., (1960) have cautiously reported that the number of knobs on the surface of Shope papilloma virus is of the order of sixty. Since these three viruses are isodimensional and have morphological units which are five- or six-coordinated, it follows from our geometrical theory that their symmetry should be icosahedral. The only numbers of morphological units (five- and six-coordinated) in the vicinity of 60 that are allowed for an icosahedral shell are 42, 72, and 92 (i.e., triangulation numbers $T = 4, 7,$ and 9 respectively). Depending on how the electron micrographs of these tumor viruses are interpreted, a triangulation number of 4, 7, or 9 can be inferred. It should be noted that for $T = 4$ considerable deformation of the observed particles has to be postulated; that $T = 7$ might be questioned because it is a skew class; and that $T = 9$ would require that less than half the particle surface can be seen.

(c) As discussed above, it is of some theoretical interest to know whether any of the three geometrical types of icosahedral classes ($P = 1, P = 3,$ and $P \geq 7$) are preferred in nature. It will thus be interesting to see if any of the higher examples of the $P = 3$ class (e.g., the structure with 122 morphological units) or of any of the skew classes $P \leq 7$ are established in the future. But it is as well to note that, in these classes, the precise distribution of morphological units on the surface would be hard to recognize since the units do not lie in rows between adjacent 5-fold vertices ("edges"), as they do in the class $P = 1$. (See, for instance, the example of 122 morphological units in Fig. 11.)

(d) The structural features observed in dried preparations in the electron microscope may not represent the lowest energy state that would exist in solution. A rather striking example of this is to be found in some recent work of Mattern (1962), on tobacco mosaic virus. He observed that the surface of the virus in his preparations showed transverse and longitudinal periodicities, larger than those known from the X-ray work, and found that his results could be explained if the protein subunits were clustered into groups of seven (6 about 1) at the surface. Only a relatively small departure in the normally uniform helical arrangement would be necessary to produce this deformation. In the case of icosahedral viruses, a clustering of the subunits in larger aggregates than hexamers and pentamers could lead to a misinterpretation of the basic substructure. One wonders whether the very marked knobs on the surface of bacteriophage $\phi X174$ (Hall et al., 1959) might not be an instance of this phenomenon.

(e) On our theory, any isometric shell which is determined by the quasi-equivalent bonding of a

large number of subunits would be expected to have icosahedral symmetry. A special mechanism such as building on a pre-formed core as in the T-even bacteriophages (Kellenberger, 1961) would be necessary to assemble identical units into a shell of another form. It is, therefore, interesting to ask whether there is any evidence for *isometric* viruses having a shell which is not icosahedral.

Two reports of bacteriophages with octahedral heads have appeared in the literature. In one case—that of typhoid phage 2 (Bradley and Kay, 1960), the authors remark that an icosahedron cannot be ruled out, and we believe that their published micrographs are, in fact, better interpreted in this way. Indeed, if, as is very likely, the negative staining method used shows less than the complete surface, an octahedron is impossible. The same criticism applies in the case of *Bacillus mycoides* phage (Tikhonenko, 1961).

CONSTRUCTION OF SHELLS

It is clear from the models in Figures 9 and 10 that any of the quasi-equivalently subdivided shells represented by the icosadeltahedra can be built from 60 T identical structure units, each bonded in a similar way. The deformation of the bonds in quasi-equivalently related environments is quite small for the shells with icosahedral symmetry. There is, however, still one element of realism lacking in these geometric models since any of the possible icosahedral shells can be built using the same structural unit. By contrast, when an icosahedral virus shell is built of 60 T identical protein molecules, it is unlikely that the structure unit of any particular virus can aggregate in more than one of the possible icosahedral shells. In order to achieve this in a model, bonding sites between the structure units would be required at more than one radius. When the units aggregate, they would bond together at a characteristic angle to each other. Thus, they possess a property we might call "built-in curvature" to form a shell of the right size.

We have shown (Caspar and Klug, 1963) how a model for a structure unit can be designed which can be assembled into only one of the possible icosahedral shells. Figure 12 shows a model for the shell with triangulation number $T = 3$, which is built of 180 asymmetric shaped wooden structure units. The structure unit has bonding sites on it which, purely geometrically, tend to form a plane hexagonal lattice, but which, at the same time, require that the units aggregate in a curved surface. The unique feature of this model is that the subunits can be assembled in only one way to form a stable shell.

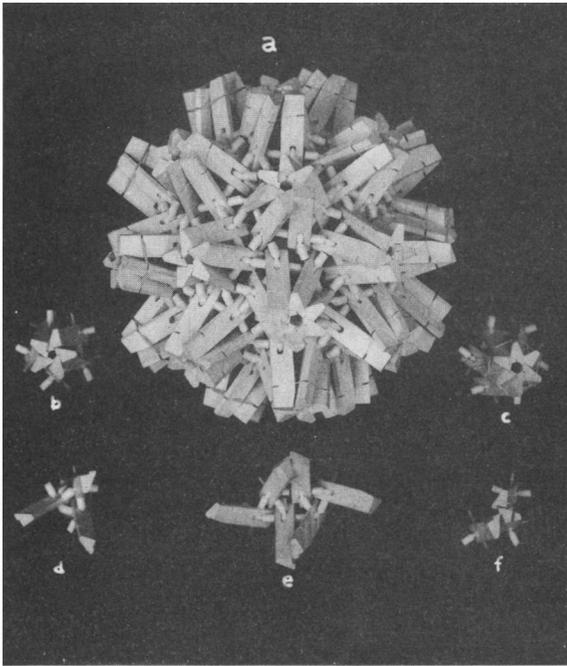


FIGURE 12. (a) A "dynamic" model (Caspar and Klug, 1963) of 180 structure units arranged with icosahedral symmetry ($T = 3$). (Cf Fig. 9). The units have been so designed that they can only assemble in one way to form a stable shell.

The particular bonding pattern chosen leads automatically to a clustering into 20 hexamers and 12 pentamers. The number and arrangement of the 32 clusters corresponds to that of the morphological units observed in turnip yellow mosaic virus (Huxley and Zubay, 1960; Nixon and Gibbs, 1960).

Various small aggregates of units are also shown: (b) pentamer; (c) hexamer; (d) trimer; (e) two trimers bonded together; (f) part of a hexamer or pentamer.

THE MECHANICS OF SHELL FORMATION

The driving energy for forming a closed shell is provided by the inter-subunit bonds. More bonds can be formed in the closed shell than in any unclosed surface array built up of the same number of subunits. As we have shown, the only efficient way in which a shell can be constructed from a large number of identical units requires that these units be designed to bond together in a doubly-curved hexagonal lattice. In the lowest-energy bonding state between an isolated pair of hexamers, the hexamer axes will be at an angle to each other. This is a consequence of the built-in curvature we have just discussed.

It is topologically impossible to build a uniformly curved surface only with hexamers. Thus, if hexamers only are bonded in a sheet, it must necessarily deform toward a plane. This deformation will strain the inter-subunit bonds relative to their lowest energy state, and the deformation energy will increase as more hexamers are added. At some point, the deformation energy will exceed

the bond energy, and further growth of the sheet would be unstable. The only way in which the natural curvature required by the subunit bonding pattern can be realized is to introduce pentamers. The most stable arrangement will be that in which the twelve pentamers topologically required to form a closed shell are as symmetrically disposed as possible—that is, in a shell with icosahedral symmetry.

The lowest energy bonding between structure units will require a certain mean radius of curvature for the surface aggregate. The ratio of the distance between a pair of hexamers to this radius will approximate the ratio of edge length to mean radius of only one of the geometrically possible icosadeltahedra. Thus, there will in general be only one quasi-equivalent closed-shell packing arrangement of lowest energy possible for a particular structure unit.

It is geometrically often possible to arrange the 60 T units which can form an icosahedral shell into surfaces of lower symmetry. It can be shown (Caspar and Klug, 1963) that if, as we predict, icosahedral viruses are built of subunits which can assemble themselves, then the most probable mistake in assembly that is likely to occur would lead to tubular forms. Tubular structures which have a diameter and surface structure similar to icosahedral virus particles have been observed associated with polyoma (Howatson and Almeida, 1960) and papilloma viruses (R. C. Williams et al., 1960; Breedis et al., 1962).

THE QUESTION OF THE INDEPENDENT EXISTENCE OF MORPHOLOGICAL UNITS

It is not necessary, in the description of the process of assembly, to postulate the existence of preformed hexamers and/or pentamers to complete the shells. The lowest energy bonding state for a small number of structure units may be either a dimer, trimer, pentamer, or hexamer. There may be many cases where the hexamer is preferred.

The morphological units observed in highly subdivided shells are presumably hexamers, together with twelve pentamers. The morphological units persist in broken down shells of viruses, such as Herpes (Wildy et al., 1960) and pseudorabies (Reissig and Kaplan, 1962). In disrupted papilloma virus shells (Breedis et al., 1962) completely separated morphological units—most likely hexamers—have been observed. Thus, for some viruses, isolated hexamers are evidently stable enough to exist independently. Whether or not the isolated hexamers are stable is not particularly relevant to the way in which a stable shell is constructed. In the closed shell there will also be dimer and trimer interactions between structure units. As pointed

out above, the closed shell design allows the formation of the maximum number of bonds between structure units, and thus structure units which can aggregate in a curved surface will necessarily tend to assemble themselves into one of the possible icosahedral shells. There is no necessity to pre-assemble the structure units into hexamers or any other small aggregate, though this may occur naturally. If the hexamers are particularly stable they might be preformed, but when they are assembled into the shell, twelve of them would be transformed into pentamers. This will happen even if the isolated pentamers are themselves unstable, since this is the only way a stable shell can be realized.

MULTISHELL AND STATISTICAL ARRANGEMENTS

A number of variations of the same basic quasi-equivalently subdivided icosahedral shell design are possible among the different icosahedral viruses. For example, some might have shells built of two or more layers of protein subunits, but each layer could still be constructed according to these same principles of efficient design. The structure unit itself could consist of two or more chemically different protein molecules. The model for the structure of an icosahedral virus shell proposed here is one in which all structure units are not only identical, but all make the same bonds. The structure unit is, therefore, monofunctional and is synonymous with the building unit.

It is possible, however, to imagine a different kind of building unit (which would now be synonymous with the morphological unit) which is multifunctional, i.e., it is fitted with different sets of bonds capable of being used in different non-equivalent situations on a highly subdivided polyhedral shell, for instance, in a face, on an edge, or at the vertex of an icosahedron. It is a fairly straightforward matter to enumerate the minimum number of "bonds" required on various criteria of packing. For units that can be assembled into an icosahedral shell, these "bonds" must be arranged at certain more or less fixed angles, and the minimum requirement is that they are present in six equivalent sets. The building unit is, therefore, likely to be a hexamer, which tends to aggregate in plane sheets, but this hexamer could also be bonded (less perfectly, perhaps) at a vertex or edge of the icosahedron. Any shell built in this way could not have true icosahedral symmetry (since there is a hexamer placed where a 5-fold axis should be), but if the building units were put in at random into the different orientations, the structure might be said to possess *statistical* icosahedral symmetry.

The most important limitation of statistically bonded building units is that they can be assembled in an indefinite number of different ways. To construct a shell of definite size, they would have to be assembled on a preformed core. For example, statistically bonded hexamers could be added to the outside of an icosahedral shell built of 60 T identical, quasi-equivalently bonded units, to form a second layer on the surface. This inner shell would, however, have to be able to build itself in only one way. The statistically bonded units alone could not build a definite structure.

SHELLS AND MEMBRANES

The largest icosahedral virus yet identified is *Tipula* iridescent virus (Williams and Smith, 1958) which has been reported to consist of 812 morphological units (Smith and Hills, 1960). Thus the value of T is presumably equal to 81, and we would therefore expect that the protein shell is constructed from 81×60 structure units. It will be interesting to see, when there are enough experimental data, whether there is an upper limit to the size of an icosahedral shell that can be efficiently constructed. In this connection, the "largeness" that is structurally relevant is the number of units in the shell and not its physical dimensions. It is possible to design an icosahedral shell that can be constructed from 60 T identical quasi-equivalently bonded units, where T is any number given by the geometric selection rule. However, when T is very large, such a shell might not be able to assemble itself in only one way. The reason for this (Caspar and Klug, 1963) is that with very highly subdivided shells, there are a number of closely related designs which can be built with nearly the same number of units, and which differ very little in energy.

Large containers could be constructed from identical units quasi-equivalently bonded into a large number of 6-coordinated units (themselves probably hexamers) together with twelve 5-coordinated units (either pentamers, or statistically bonded hexamers). Such a container, constructed of globular units would be thin, compared to its diameter, and would thus be intrinsically more flexible than a shell built of a smaller number of units.

It is apparent that the structural distinction between a rigid shell and a flexible membrane is not sharp. The shape of a flexible membrane is more likely to be determined by its contents than by considerations of the lowest energy arrangement of the structural units. The 5-coordinated units topologically required in a membrane could be statistically arranged, whereas in a more rigid shell, capable of self-assembly, the pentamers would be

arranged with icosahedral symmetry. The important point for the construction of any closed container is that the most efficient designs are all based on a folded hexagonal net. The same general type of substructure might be expected in cellular membranes, as in icosahedral virus shells. The distinction is that a shell is relatively rigid and only a fixed number of structure units can assemble themselves in one efficient way to form the shell; on the other hand, a flexible membrane is capable of growth and its size is determined by its contents.

SELF-ASSEMBLY OF ICOSAHEDRAL VIRUSES?

EMPTY SHELLS

Empty shells are found associated with most icosahedral viruses. As we have shown (Caspar and Klug, 1963), the subunits of an icosahedral shell constructed according to principles of efficient design would be able to assemble themselves without the need of a core or any external "organizer". So far, no icosahedral virus shell has been re-assembled *in vitro* from isolated subunits. Empty shells have, however, been produced *in vitro* by leaching out the nucleic acid from some intact viruses. The alkaline degradation of the RNA of turnip yellow mosaic virus particles (Kaper, 1960) leaves behind empty shells, which have been shown to be structurally intact (Finch and Klug, 1960). Cooper (1962) has shown that the RNA of poliovirus can be released in concentrated urea solutions, without completely breaking down the protein shell. On occasions, the poliovirus RNA remained infective and therefore presumably in one piece. Similarly, heat treatment of bacteriophage ϕ X174 (MacLean and Hall, 1962) expels a fibrous core, leaving the bulk of the outer coat more or less intact. In most cases where empty virus shells are observed in the electron microscope, it is not possible to decide whether those were produced by loss of nucleic acid from intact particles, or by spontaneous aggregation of the protein subunits in infected cells. In some cases, there is suggestive electron microscope evidence (for example, Morgan et al., 1959; Smith and Hills, 1959; Epstein, 1962) that empty shells are an early developmental stage in virus particle assembly, implying that the nucleic acid may be packaged in preformed containers. In contrast, the studies on the development of the more complex bacteriophages (see Kellenberger, 1961) indicate that the protein coat is assembled on a preformed nucleic acid core. The phage ghosts observed in proflavin treatment of bacteria appear to be defective particles which have lost their DNA and do not represent early stages in the assembly

process. The non-infectious poliovirus particles which are produced in the presence of proflavin (Ledinko, 1958) may be similarly defective.

If it can be established that polymerization of protein subunits into empty shells proceeds spontaneously in cells infected with an icosahedral virus, it should be possible to reproduce this process *in vitro*. Moreover, this would be a convincing demonstration that icosahedral viruses are constructed according to the design principles we have proposed.

The strongest evidence for the self-assembly of an icosahedral virus shell is provided by the recent study by Reissig and Kaplan (1962) of the non-infectious particles produced by 5-fluorouracil-treated cells infected with pseudo-rabies virus. The 5-fluorouracil blocks DNA synthesis and the non-infective particles produced lack the electron dense core characteristic of infectious particles, though they have the same external appearance as the intact virus. This is presumptive evidence that the non-infectious particles contain little or no nucleic acid. Since the DNA synthesis of the cell was blocked, it is unlikely that these empty shells were produced by losing a transient DNA core. It thus appears that these shells were produced by spontaneous aggregation of protein subunits in the infected cell.

ROLE OF NUCLEIC ACID IN ICOSAHEDRAL VIRUS STRUCTURE

Even if the protein shell can form without the virus specific nucleic acid, this does not mean that the nucleic acid has no structural function in forming the virus particle. It is appropriate to consider the example of the helical tobacco mosaic virus particle in this connection (see Klug and Caspar, 1960). The isolated protein subunits can be polymerized *in vitro* in the same helical arrangement as in the intact virus, but the polymerized protein rods are much less stable than the RNA-containing native or reconstituted virus rods. Moreover, under some conditions the protein subunits alone can be polymerized in a "stacked disc" structure which could not accommodate the RNA. Thus, the nucleic acid contributes to the stability of the virus particles and prevents "mistakes" in the assembly. It is important to remember, however, that it is the packing properties of the protein which determine both the structure of the particle and the configuration of the nucleic acid in the intact virus.

The properties of the empty shells and intact particles of papilloma virus (Williams, Kass, and Knight, 1960; Breedis, Berwick, and Anderson, 1962) show a similarity to the properties of the nucleic acid-free and intact TMV particles.

Partially purified papilloma virus preparations can be separated into fractions according to density. The dense bottom layer has the highest DNA content and infectivity, and appears, in the electron microscope, to consist of intact virus particles. The least dense layer has little DNA, low infectivity, and appears to consist largely of empty particles, as well as occasional aberrant tubular forms. As previously mentioned, these tubes presumably represent mistakes in subunit assembly and their existence suggests that the subunits are designed to assemble themselves. Breedis, Berwick, and Anderson (1962) have shown clearly that the empty particles and tubes are less stable in high concentrations of cesium chloride than the intact virus particles. Thus the DNA of papilloma contributes to the stability of the icosahedral particles. Moreover, the DNA does not appear to be accommodated by the tubular forms.

It is impossible to decide, yet, if the intact icosahedral viruses are constructed by assembling the protein subunits around the nucleic acid core, or by packing the nucleic acid in the preformed shell. TMV particles are formed by a kind of co-crystallization of the nucleic acid and protein. The RNA cannot be inserted in the preformed helical shell, nor can it be extracted from the intact virus without breaking down the helix. However, since the nucleic acid of some icosahedral viruses can be extracted without breaking down the shell structure, the assembly of the complete particle may involve the reverse of this process.

The structural problem of folding up a flexible single-stranded RNA or DNA molecule inside a shell is clearly different from the problem of folding up a relatively stiff DNA double-helix. The fact that the same type of shell design is used by viruses containing double-helical DNA and single-stranded RNA is convincing evidence that the icosahedral shell design is dependent on the packing properties of the protein subunits and not the folding properties of the nucleic acid. A flexible RNA molecule could be rolled up like a ball of string inside the shell, but the limited X-ray evidence (Klug and Finch, 1960; Longley and Klug, unpublished) indicates that the RNA folding is, in fact, related to the structure of the shell though it is not as

highly organized as the protein packing. A double-helical DNA molecule, on the other hand, cannot easily be rolled up, and is more likely to be sharply folded at sequence-determined bending points. At a bend, the regular double-helix hydrogen bonding must be disrupted. If the hydrogen bonding is weak where the molecule should fold, or if these polynucleotide segments bond preferentially to the protein, the bends could, in effect, be genetically determined. In this way, the virus DNA molecule might be selectively designed to fit in its proper shell. Other DNA molecules might just not be able to fit into the virus shell.

COMPARISON OF ICOSAHEDRAL AND HELICAL FRAMEWORKS

We have seen that two quite different types of regular container can be built out of equivalently or quasi-equivalently related subunits, namely, the helical and icosahedral. There is, therefore, an element of arbitrariness still left since theoretical considerations of shell design alone are not enough to indicate any preference of one over the other. The two designs, however, imply distinct physical differences in their properties of stability, assembly, and disassembly, so that one or the other could be selected according to the biological functions required. The properties are summarized in Table 2.

The helical framework is a natural choice for packing a long single-chain nucleic acid molecule, since it allows the maximum interaction between the nucleic acid and protein. The reason for this is that the long molecule can be wrapped up with a helical symmetry consistent with that of the protein framework. However, it appears to be impossible to wrap any long-chain molecule into a configuration with strict icosahedral symmetry. Thus, there is no way in which it can make the same kind of contacts all along its length with the protein subunits of an icosahedral shell (Klug and Finch, 1960). We would expect that maximum interaction of the nucleic acid with protein would mean better stabilization, and in support of this may refer to a discussion by Bawden (1959) of differences in the ease of inactivation of various simple plant viruses. He concluded that the arrangement of protein in

TABLE 2. HELICAL AND ICOSAHEDRAL STRUCTURES CONTRASTED

Helical	Icosahedral
Assembly process straightforward	Assembly process not necessarily in one way
Maximum regular interaction of nucleic acid with protein	Not all the nucleic acid interacting equally with protein
Particle has large surface area exposed to environment	Minimum surface area exposed to environment
Disassembly has to be total to release nucleic acid	Nucleic acid may be expelled from shell

the rod-shaped viruses seems better adapted than that of isometric particles to protect the enclosed nucleic acid.

The assembly of protein structure units into a helix is a straightforward process. There is only one way in which units can be added—thus the assembly follows a fixed path. This property of unambiguous assembly is not altered in the co-crystallization with nucleic acid to form a helical package. On the other hand, the process of assembly of units into an icosahedral shell, as we have described it here, is not so direct. It is possible to go through a large number of configurations and to make mistakes which have to be rectified before a complete shell can result. In fact, the system does not reach its lowest energy state until the shell is completed. The process may be likened to the building of an arch, which is not stable until the keystone has been put into place. Here, however, the nucleic acid may play a positive role in the dynamics of the formation of the shell, and in selecting the “correct” shell out of a number of possible alternative structures (see above).

The icosahedral shell has, however, distinctive advantages in other directions. First, the minimum amount of protein is used for the packaging of a given quantity of nucleic acid. Second—and probably more important—the surface area exposed to the environment is also a minimum, whereas a helical structure leaves a maximum area exposed. Some viruses combine features of both types of design by folding their helical nucleoprotein core into a “ball” enclosed in another coat.

There is also a marked contrast in the mode of disassembling the two types of structure, which may have important implications for the process of penetration into the host cell. A helical nucleoprotein package has to be more or less completely taken apart to expose the nucleic acid, whereas, in an icosahedral shell, the nucleic acid may be released or expelled without a disassembly of the particle.

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