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Form determination of subcellular biological structures as for example viruses

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Abstract. Morphogenesis as one of the natural mysteries is investigated by starting to understand the mechanism of form determination governing subcellular elements that are assemblies of identical subunits. We start with the simplest viruses, where the properties of the protein-subunit determine the shape of the final product. We then pass to those viruses, where the shell of the precursors is made with help of a scaffold. We discuss the proposed models of size determination and compare them with the available experimental data, particularly for the bacteriophage tail. The discussed mechanisms are likely to be valid for many subcellular elements, but not for the form of multicellular assemblies. We emphasize that embryology is under transcriptional control, in contrast to virus particle formation. We then shortly discuss the additional controls likely to function in the form determination, e.g. of multicellular organs.

1. Introduction

The species specific shape of plant and animals has challenged curiosity and research efforts of mankind since memorable times. The transmission of shape from the parents to their offspring is the basic expression of what we call inheritance. Shape is maintained through as many generations as we can observe and as we are told from our ancestors and their drawings before. Only very few variations of shape occurred during the lifespan of human civilisation.

In this century the basic mechanisms governing genetics have been discovered and are presently used to understand more about biological phenomena. Still the form determination of higher animals or plants is far from being understood, although we have an enormous bulk of observations of what we call morphogenesis. The form of multicellular organisms must be due to cell to cell interactions. Morphogenesis, therefore, must be based on changes of these interactions, of which one presently tries to understand the underlying genetic mechanisms.

It is of sound scientific practice to proceed from the simple to the more complex. Bacterial viruses have been the tool for investigating genetics. By using this experimental system one had achieved the enormous progress in understanding the basic genetic mechanisms. This development has in parallel also led to a basic understanding of the physiology of virus multiplication, virus-assembly, and virus-maturation. Although not being comparable to embryology, phage development might give us some clues to understand very simple form determinations in nature. Indeed, while it is quite certain that morphogenesis in embryology is

under transcriptional control, in viruses we know for certain that the underlying mechanisms are based only on protein–protein and protein- nucleic acid interactions. The morphogenesis of viruses is thus not an example for embryology, but rather for the morphogenesis of subcellular elements like cytoskeleton and cellular organelles.

Viruses are constituted of a coiled nucleic acid filament which is protected by a shell made of proteins. Some viruses have an additional outer layer made of proteins and lipids. Most viruses have a strikingly constant form, and each species keeps this form independent of environment and growth conditions. This form is related to simple geometrical bodies, like icosohedra and cylinders. The nucleic acid molecule contains the information necessary for the multiplication of the virus in its specific host. At least one of its genes, frequently more, determines the protein(s) of the shell. When Watson and Crick discovered the structure of DNA and postulated the genetic code that is used to convey the information of each gene, they immediately realized that viruses have to be built from many protein subunits. Indeed the size of a protein as a final product of a gene, is much too small for covering and protecting entirely the nucleic acid. One therefore classifies viruses according to the number of protein subunits they contain, and this in respect to the number of protein species as well as of the number of identical proteins involved.

2. The first level of complexity: Assemblies of fully shape determining subunits

Tobaccomosaic Viruses (TMV) belongs to one of the simplest viruses known. It is filamentous, that is its capsid is built as a cylindrical assembly of identical protein subunits. The protein and the nucleic acid of TMV can easily become separated and individually purified. It was soon discovered that the protein subunits by themselves are already able to assemble into a cylinder. Obviously, this "empty" virus particle is not infectous because it lacks the RNA, which is the carrier of information. Since we deal only with one species of protein, and this protein is able to assemble into one given geometric structure only, we have to conclude that each subunit "knows" how its final product of assembly has to look. In other words, the protein subunits are shape determining. This ability to determine the shape of the assembly product (or aggregate) can only be explained by the binding properties and geometry of these subunits (Fig. 1). Soon after the discovery of the genetic code, Perutz and Kendrew found out that proteins, as the final products of a gene, have very specific geometrical conformations. The conformation of the folded polypeptide chain is determined through the aminoacid sequence, which is encoded in the gene. It was shown, with TMV virus, that mutations in the nucleic acid resulted in alterations of the aminoacid sequence of the proteins. Frequently this protein lost the ability to form capsids and active viruses. In some particular cases, where one selected for them, it was found that the morphology can become altered: Instead of forming compact cylinders, as with normal TMV, some scientists found different sorts of helices of a more or less open structure [1, 2]. A naturally occurring strain (Dahlemense-strain) also shows distinct morphological differences and had been studied extensively [3]. But already the normal subunit is able to assemble into slightly different types of rods, according to conditions prevailing during assembly ([4], and Fig. 1a

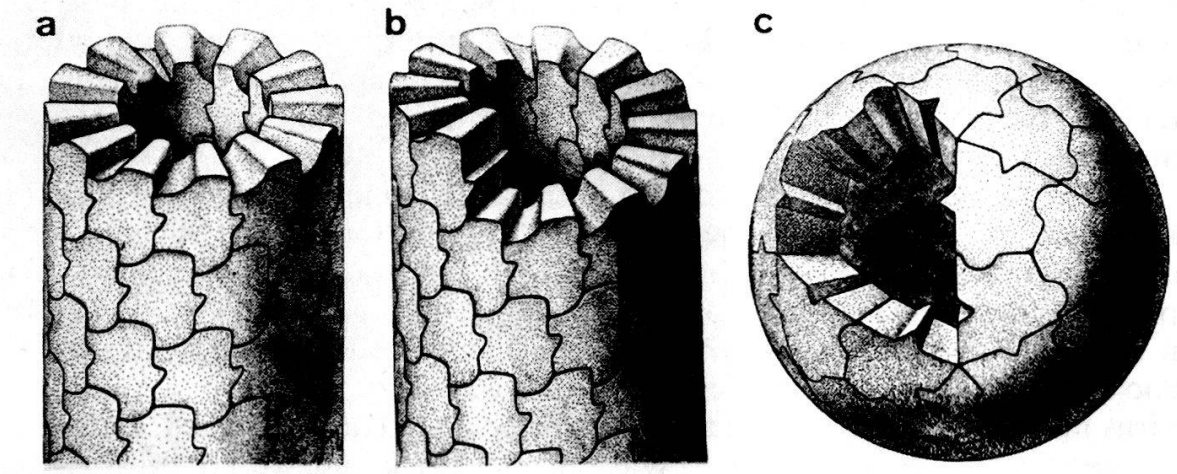

Figure 1
Cylindrical and spherical assemblies made of identical subunits
The reality is simplified by assuming only a purely geometric fit, like in the case of a "puzzle". Figures a and b exemplifies two conformations, (a) stacked disc and (b) helical as they occur e.g. with tobacco mosaic virus (TMV). In the sphere the 60 subunits have perfect icosahedral symmetry. In each of the 12 vertices of the generating icosahedron 5 subunits are placed with 5 fold rotational symmetry. (From Sci. Am. [26].)

and b). What we have to retain from these studies for the purpose of the present considerations, is that protein subunits are very often shape determinant for the aggregate which they form.

It was shown very early that spheres can also be built from identical subunits when assuming icosahedral symmetry (Fig. 1c). To build a sphere we need at least 60 subunits which are clustered into pentamers. In this situation we have completely equivalent positions of each protein subunit with respect to each other of the same virus.

These geometric considerations were pushed further in the classical work of Caspar and Klug [5] in which they showed that larger, closed particles related to icosahedral symmetry can be built when assuming the principle of quasi-equivalence. Today we understand that quasi-relevance is the requirement for slight modifications of the individual morphologies of the protein subunits according to their position on the geometric body [19]. They are slightly different, when situated in the faces, edges or vertices of the icosahedron. The symmetry is no longer completely fulfilled, but only approximately. Essential for this fundamental concept is the close contact between subunits, ascertaining the mechanically required, relatively strong interactions. The interactions are the sum of so-called weak (non covalent) interactions that occur in the area of contact. It is interesting to note that the concept of quasi-equivalence infers automatically plastic or elastic properties of proteins (Fig. 2).

Many spherical viruses were studied and for most of them the separation into nucleic acid and protein was found to be easily feasable. In many cases nucleic acid free shells could be assembled out of the protein subunits. The form determining property of the protein subunit was demonstrated here also. However, in many cases the assembly product was no longer of clearly defined shape

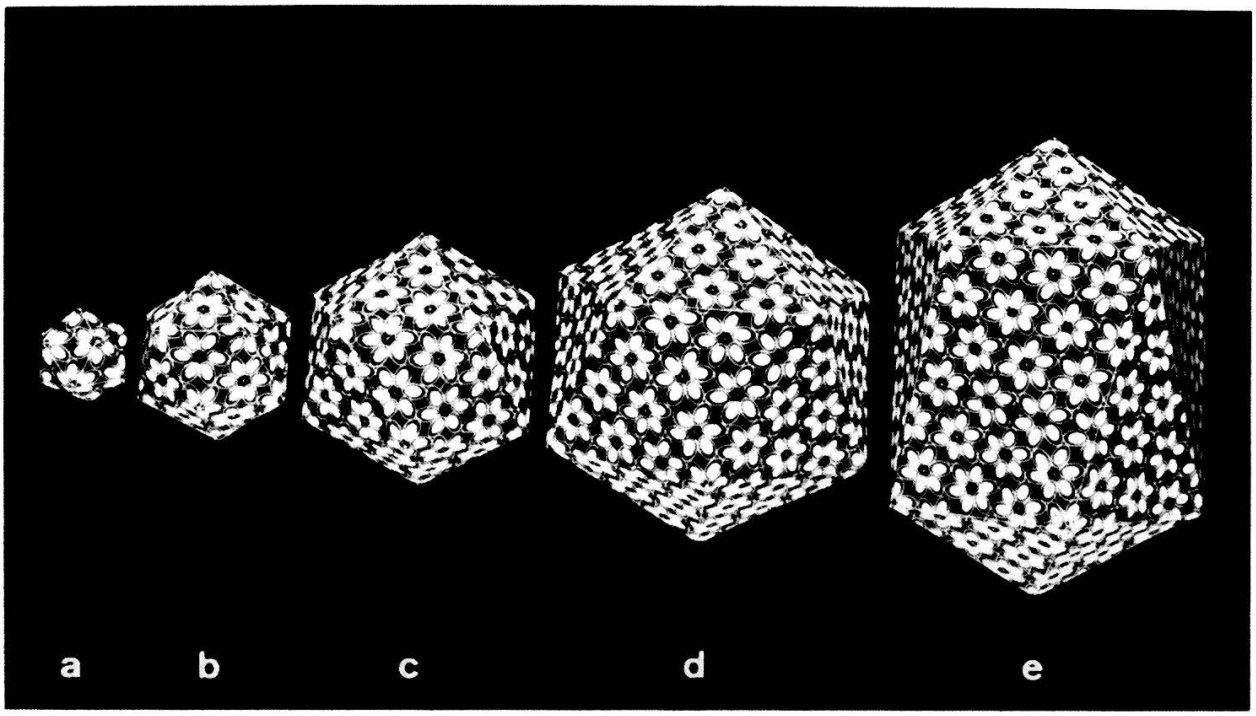

Figure 2
Models of icosahedral bodies
(a) corresponds to Fig. 1c with 60 subunits ($T = 1$). The number of subunits is calculated from the triangulation number T as $N = 60T$, or $N = 30(T + Q)$.
(b), (c) and (d) are $T = 3$, 7 and 13. They require quasi-equivalence of the subunits as explained in the text. This is related to groups ("capsomers") of 6 situated on the faces, and of only 5 in the vertices.
(e) is the prolate derivative of (d). The "equatorial band" of the icosahedron is enlarged by a number of subunits that is expressed by Q.
The head of bacteriophage λ is an icosahedron with $T = 7$, that of phage T4 a prolate icosahedron of $T = 13$ and $Q = 21$ or possibly 22. (Models by courtesy Dr. M. Wurtz.)

and something must have gone wrong. This brings us to the second level of complexity.

3. The second level of complexity: The need for scaffolds

Bacterial viruses were particularly well studied because the experimental system, based on bacteria as hosts allows rapid experimentation. It is easy to cultivate bacterial cells, to infect them under controlled conditions, and thus to get reproducible results. It was soon discovered that here also, as with animal and plant viruses, we find RNA and DNA containing viruses. It was then found that most of the double-stranded DNA containing viruses studied had a reproductive cycle in two steps: After infection and replication of the nucleic acid a precursor is made which consists only of proteins. This provirion or in bacteriophages, prohead will then, in a further step, mature into the final virion or head. This maturation involves profound modifications: the shell expands and the DNA is taken up ("packaging"). Most relevant was the discovery that these proheads always contain internal material in the form of what is now called a scaffolding core. The cores of many bacteriophages are known with respect to their protein composition.

With bacteriophages so-called conditional-lethal mutants are easy to achieve [6]; they are widely used for investigating these proteins. Mutants of the core proteins were found which, *in vivo*, lead to an aberrant morphology of the shell. In reproducing the results *in vitro*, as has been done with TMV by starting from isolated proteins, it was found that the shell protein itself is not able to give a high yield of normally formed and sized capsids. If, however, the scaffolding proteins are also present, the exact form of a provirion or prohead can be reproduced *in vitro* [7, 8]. One has deduced from these findings that the core proteins have indeed a form determining role: they help to determine the final shape of a virus shell. The protein subunits of the shell are now no longer *fully*, but only *partly*, shape determining; additional shape determining information is contributed through the core proteins. The amount of information conferred by each of the two determinants is variable from species to species and is still, in many cases, the matter of investigations and speculations [9].

It may be noted here that the proteins of the scaffolding core are in general removed or at least partly digested before or during packaging. In some cases the core proteins can be reused for building new proheads within the same bacterium [10]. It is still a matter of conjuncture how much the core proteins are involved in the uptake of DNA during maturation. For some viruses it was demonstrated that only the "empty", i.e. core-free proheads are able to act as substrates for *in vitro* packaging [11].

While the *in vitro* experiments showed that the subunits of the shell proteins alone do not assemble into the correctly shaped particle, the reverse was found to be true for core proteins. It was indeed possible to reassemble T4-core proteins *in vitro* [12] into cores of exactly the same shape as is formed *in vivo* [13]. There is no doubt that the scaffolding core has already its own shape determination.

Figure 3 illustrates this situation for the bacteriophage λ; we see the normal

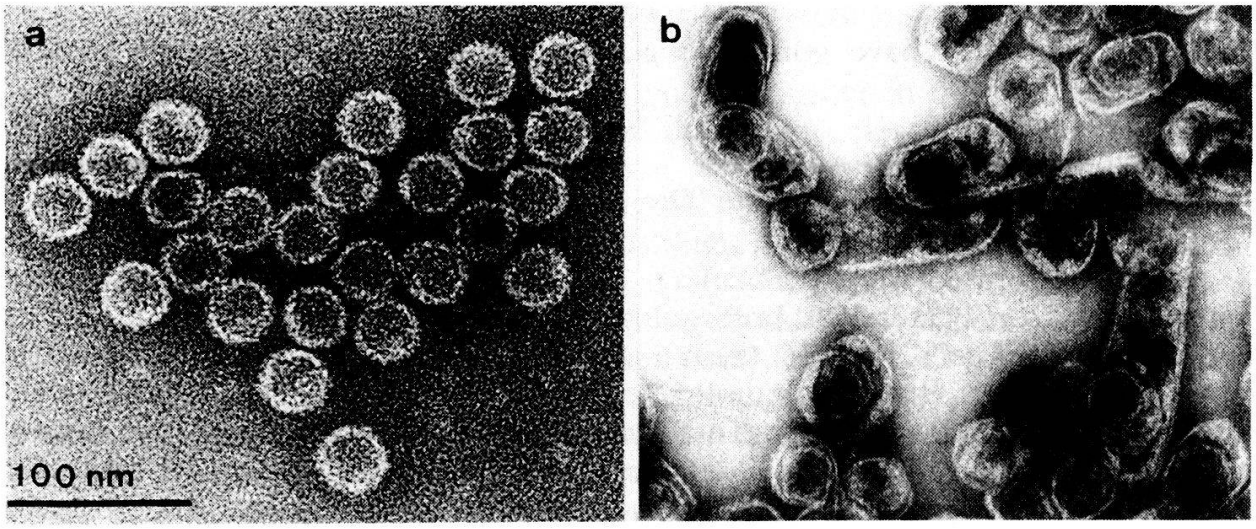

Figure 3
The need of a scaffolding core for producing normal λ-proheads
(a) shows proheads from which the core has already departed. They are ready for DNA packaging. (b) shows proheads produced by a phage which is mutated and does not produce a functional core-protein (Nu 3^-). It produces only aberrant assemblies of the head-shell-protein, which cannot become packaged with DNA. Prepared in negative stain. (Micrographs by Dr. M. Wurtz.)

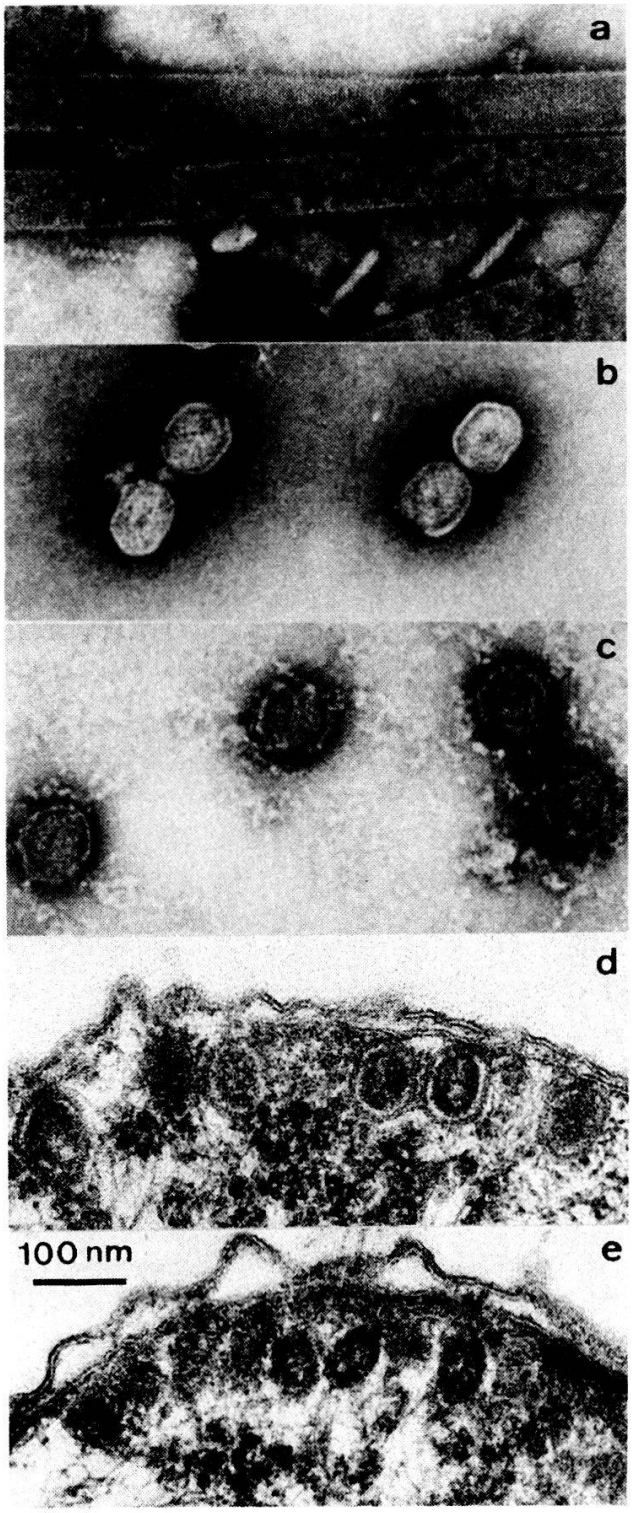

Figure 4
Aberrant and normal head precursors of phage T4
(a) shows the head-shell-subunits assembled into tubular forms. This happens when core proteins are lacking. Two tails are also visible.
(b) shows normal T4 proheads with their scaffolding core, produced *in vivo*.
(c) *in vitro* reassembled proheads. They are surrounded by some random aggregates of the subunits.
(a), (b) and (c) preparations and micrographs by R. Van Driel and E. Couture [8, 12]. Prepared in negative stain.
(d) and (e) are unpublished thin sections from the work of F. Traub with M. Maeder [13].
(d) shows normal proheads in longitudinal cross-section. The core and shell are clearly visible.
(e) shows naked cores which are obtained when no functioning shell protein is produced.

proheads in (a) and in (b) the abnormally shaped proheads which are assembled from shell proteins in the absence of the core protein Nu 3.

Bacteriophage T4 is particularly suitable for the study of form determination. It is already on a slightly higher level of complexity: its form is not a simple icosahedron but an elongated so-called prolate icosahedron. Through geometrical considerations it is very difficult to imagine a protein subunit which is fully shape determining and which is able to build a prolate-shaped head. Here the role of the core seems to be particularly needed. In Fig. 4 we see some of the features of this bacteriophage system. In the absence of core proteins, particles of tubular shape are produced, the so-called polyheads (Fig. 4a). But in the absence of other scaffold proteins abnormal shapes of more or less rounded particles are observed. Naked scaffolds without shell can be observed *in vivo* [13] and produced *in vitro* [12] (Fig. 4c–e). They have the typical double-conical shape, as is found also within *in vitro* or *in vivo* produced proheads (Fig. 4b–d). This bacteriophage has a peculiar property (Fig. 5c): It was discovered that variants can be produced which have either a shorter or a longer head [14]. These isometric and giant particles are seen in Fig. 5a, b, d. They are never produced as a major part of a progeny

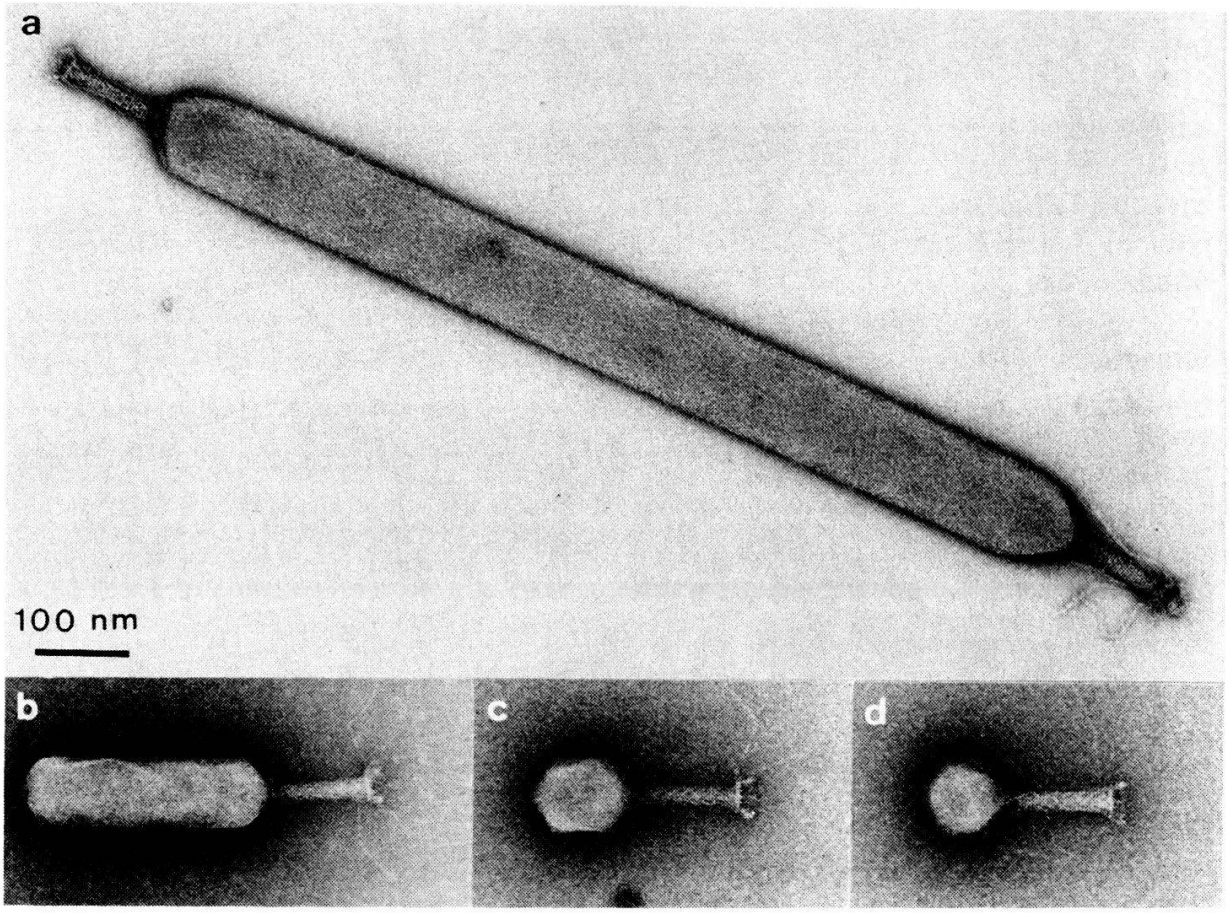

Figure 5
Bacteriophage T4 with different head lengths
(a) giant with two tails
(b) smaller giant
(c) normal prolate head
(d) isometric head ($T = 13 = Q$)
Micrographs courtesy of E. Boy de la Tour (Geneva) and F. Eiserling, when in Geneva and Basel.

population but only as a few percent, reaching at the most 50% in the case of isometric particles. Most puzzling, however, was the finding that such form variants can be produced either by mutations affecting the shell protein or by mutants affecting the scaffold and even when growing normal phages in some particular hosts (for reference see [9]). One has been able to show that proheads of the form variants have the same features; they undergo the same pathway of maturation. This reduces the problem again to that of the form determination of the prohead. Work is in progress in different parts of the world on evaluating the reasons and mechanisms for this type of form variation; we will discuss them at the end of this paper.

4. The length determination of the phage tail

In Fig. 6 we see the schematically summarized results of investigations of bacteriophage T4, which show that the assembly of the final, infective virion is

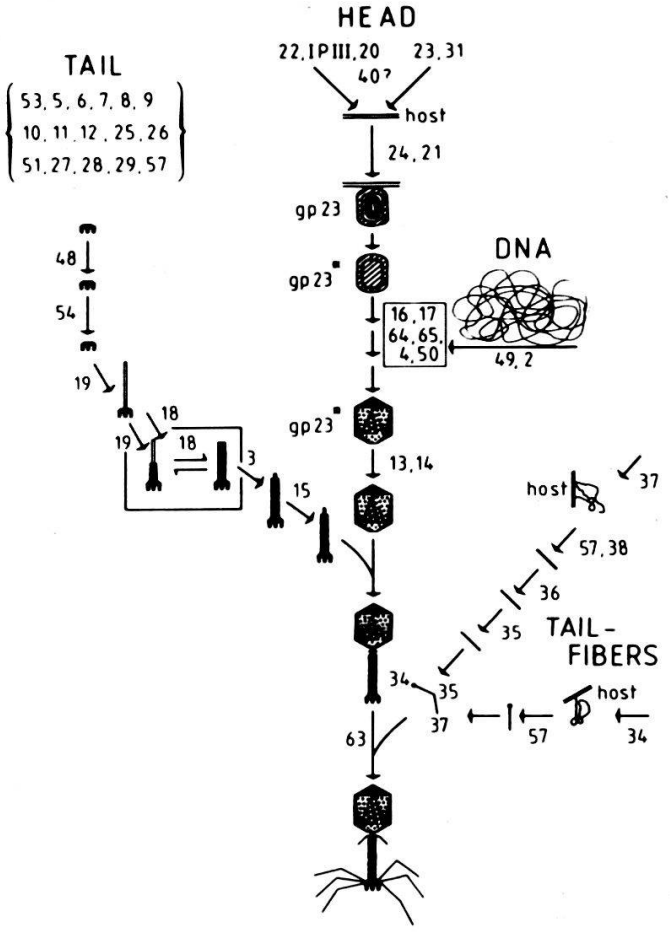

Figure 6
The assembly lines of bacteriophage T4
According to Wood et al. [27], modified and complemented according to recent data of different laboratories. The numbers besides every step designate the gene of which the product is involved. In most cases the lack of a functional gene product leads to arrest at this step. In a few cases, but particularly with the head, side tracks develop which lead to abnormal, abortive particles, like e.g. the tubular "polyheads" (see Fig. 4a) or polysheaths.

composed of four independent assembly pathways. One concerns the phage tail, one the phage head, and two additional ones the tail fibers. All three eventually come together and produce the active, infective particle. We have already considered some of the features of the head pathway; we will now concentrate on some particular features of that of the phage tail. One of these is the surprising fact that the phage tail is always exactly the same length and therefore consists of the same number of subunits. But it was also found that it is practically impossible to produce length variations of this tail through mutations. Phage tails became particularly suitable model-systems for exploring length determination which is obviously of highest interest in biology when remembering the muscle fibers with their regular periodicities.

Tail assembly was particularly studied by the laboratory of Jonathan King, in the USA, for bacteriophage T4 [15] and by A. Katsura, in my laboratory, for bacteriphage λ [16]. It turned out that tail assembly is straightforward and does not involve any maturation steps as is e.g. the case with the head. In tail assembly, one protein after the other becomes added to the structure as is seen in Fig. 7. If one gene product is not available as an active protein, then assembly is stopped exactly at this step and the previous structure accumulates. Some complications might occur also here: The lack of a gene product might lead to abortive assemblies as e.g. with T4 to polysheaths, a variant of the tail sheath which occurs when no tail tubes are available, or with λ to aberrant, very long tails.

In the length determination of the bacteriophage T4 tail the central tube or core plays an important role. When assembled base plates are put in the presence of tail tube subunits (gp19), then the correct length of the assembly is automatically achieved. The question arose very rapidly about the mechanism which determines this precise length of 122 gp19-subunits. As we will discuss further below, several

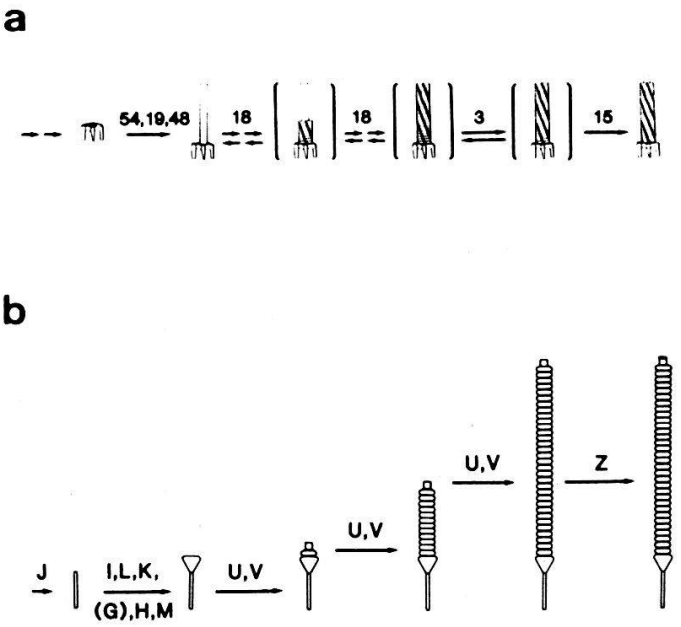

Figure 7
The tail assembly pathways
(a) Tail of phage T4, simplified from King et al. [10].
(b) Tail of phage λ, simplified from Katsura [16].

hypotheses were formulated. Presently the findings of two groups in the United States, that of Bloomfield in Minneapolis and of Eiserling in California, suggest very strongly that a single gene product is producing a fibrous protein which determines the length by acting as a ruler. This protein would be attached to the base plate and guides the assembly of gp19 around it. The end of the fiber would be the signal for termination of the polymerization. Suggestive evidence was already available for both phages [16, 17], but the final proof is very difficult to provide. It consists indeed of genetic manipulation: the "ruler", i.e. the length determining protein fiber, has to be shortened by removing part of the gene. This has likely to be done by deleting pieces from the middle part of the fiber without affecting the two terminal ends. These might play an important role. This work is under way and will, in our opinion, settle the problem of length determination which has been pending for many years.

In the further assembly of the T4 tail, the central tail tube is surrounded by a tail sheath, assembled from another protein which is now using the tail tube as length determining ruler. The interest of the tail sheath is that it is contractile and plays an important role in phage infection. We can unfortunately not discuss this problem within the frame of this article [18, 19].

5. The four proposed models for size determination as part of form determining mechanisms

In the preceding sections we have discussed two instances in which length determination is obvious. We have first seen that the bacteriophage T4 has head length variants: the normal prolate phage can be modified into a short isometric or a giant bacteriophage head. The normal and the giants are normally infective and it was shown that the giants contain several complete genomes. The isometric particle contains a reduced amount of DNA and, because the DNA in this phage is cyclically permuted, each individual particle lacks an other piece. If several isometric particles are used for infection they complement each other such that at least one copy of each gene is present.

Then we have explained the length determination of a linear structure, as represented by the bacteriophage tail. We have learned that the present state of investigations suggests very strongly that a ruler, in the form of a fibrous protein of genetically determined length, is acting as a scaffold for the length. With these two examples in mind we can now discuss different models which were proposed as possible mechanisms of length determination in morphopoieses.

The first model is characterised by the idea of a scaffold [21]. The scaffold is contributing information towards the form (and thus also size) of the shell and acts only transiently. Such a scaffolding core is found in the proheads and it was indeed demonstrated that its absence or aberration leads to abnormal assemblies, as e.g. the tubular forms. The length-determining ruler, proposed for the tail, as we have just discussed, is already a simple case of a scaffold with similar functions.

Another model for length determination was proposed much earlier and is based on the principle of the vernier, as is used on measuring instruments [22]. To understand this model let us assume subunits of two species of protein which polymerize simultaneously into concentric tubes. They have different sizes and they will therefore attain register only after a certain number of subunits have

been assembled. This event would stop further polymerization. The principle of this model, as explained on concentric cylinders, is obviously applicable to more complex situations as e.g. scaffold and shell of the prohead [20]. This very attractive model has, however, never been proven to be applied in nature. The theoretical arguments which can be brought-up against it are not sufficiently strong to rule it out.

A further model was proposed and is based on the observation of quasi-equivalence as we have discussed above. It was finally called the "model of cumulated strain" [23]. It consists essentially of the following ideas: Subunits assemble into a structure; during this assembly, they are additively distorted as a consequence of mutual interaction. The accompanying strain cumulates to a threshold at which a switch occurs to another minimal energy state of the complete structure. It can be symbolized by a change of two energy minima, as shown in Fig. 8, of which one is progressively increased so as to disappear. On the now flat potential surface a ball will fall into the next energy dip as represented on this figure. This switch would involve conformational changes of the subunits and possibly also a strong increase of the interactions between the subunits. Predictions of this model are that the terminal proteins e.g. of a cylindrical assembly must have a conformation that is different from the other ones. No definite evidence for the real existence of this model exists as yet.

The last model was proposed by M. Showe et al. [4] and accounts for several experimental observations on the influence of gene dosage on head-length variations of bacteriophage T4. It is based on the assumption that the scaffolding core and the shell of proheads grow simultaneously, in parallel. The velocity of each of these growths determines whether the prohead will be short or long: If the core assembles and thus elongates faster than the shell there will be a tendency to produce giants. If, on the contrary, the shell grows faster than the core there will be a tendency to produce short heads. The particular interest of this model is that it accounts for the fact that these form variants are always only a small part of an otherways normal particle population. First checks of this kinetic model, however, did not confirm or exclude the model. According to purely chemical considerations the velocity of assembly depends on the concentration of the respective unassembled subunits. It will be faster the more subunits are available and

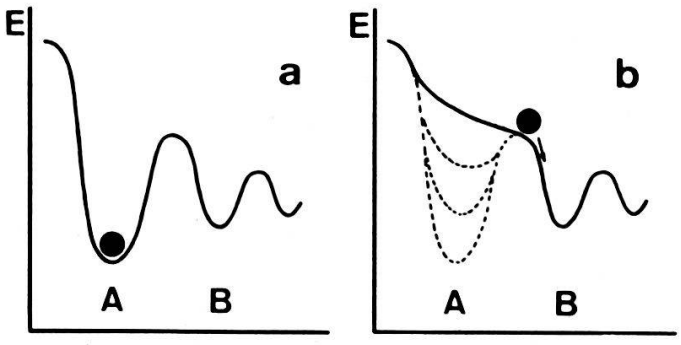

Figure 8
The model of "cumulated strain" for size determinations
Two conformational states of a protein are reflected in two potential wells (*A* and *B*). During assembly of proteins in conformation *A*, the progressively cumulated strain removes well *A* and, when all together have decreased, a switch occurs, symbolised here by the sphere falling in well *B*. At this moment, the proteins change into conformation *B* and again a very stable state is established.

synthesized. To a second degree they obviously also depend on affinities of the interactions, and these could become modified with mutationally affected proteins or by effects of the intracellular environment. The observations of T. Grütter, a graduate student in our laboratory, have shown that varying these subunit concentrations did not alter the proportion of form variants in the expected manner, but exactly in a reverse way [25].

6. Discussion and perspectives

6.1. *Assemblies of protein subunits*

We have seen that studies of the form determination of protein assemblies have still a long way to go before becoming completely understood. Nevertheless, the attempts made on simple systems have been rewarding in the sense that one now gets a clear feeling of having made progress towards the goal. As we have seen, it is likely that the mechanism of length determination of the bacteriophage tail will be demonstrated very soon. The present experimental data of Eiserling et al. (personal communication) and Bloomfield et al. (personal communication) are already rather strong.

It is much more difficult to understand the form determination of the head. Here we have the very bothersome paradox of the facts that mutants affecting either the scaffold or the shell lead to form variants. We have clear indications that the core is needed to give the correct shape to the capsid. But by this we only bring the form determination to a further level, to that of the scaffold. What are the mechanisms for determination of the length and shape of the scaffold? *In vitro* experiments hiterto have failed to produce either short or giant scaffolds. This experimental work has to be pursued and developed further. Clearly a long way is still facing us. Among other things it becomes particularly important to correctly reproduce *in vitro* the situation *in vivo*, an aspect which is in general very much neglected. Many *in vitro* experiments simulate effects, which, intracellularly, are certainly done differently. To choose just one example: the expansion of proheads, which occurs during its maturation into the final head, is produced *in vitro* for the phage T4 simply by ionic dilution, while for the phage λ one needs the action of urea. Neither will occur *in vivo*. Thus we simulate an event *in vitro* by mechanisms which do not exist *in vivo*. Indeed, *in vivo* phage maturation is not synchroneous at all, and a relatively high ionic strength (150–600 mM for K^+ e.g.) is maintained intracellularly as long as phage multiplication procedes. It is obvious also that urea is not produced in measurable amounts in infected bacteria.

6.2. *Multicellular aggregates: The third level of complexity*

Above we have mentioned the difficulties already encountered at low levels of complexity, in order to discuss and understand why it will be even much more difficult on the third level of complexity, that is on the level of multicellular plants or animals, and even on cells of tissues.

One of the most exciting observations is the fact that one can remove part of a liver of a mammal and that this liver will become reconstituted in its approximately original shape. The cells therefore must have information which, after a

certain growth, will stop their proliferation. It must act in a very controlled fashion, such that the resulting shape of the liver is correct. Understanding this requires much more knowledge on the transfer of information from cell to cell than is presently available. Fortunately, numerous people are working on these cell to cell communications. Very much more will also be understood if we have a better knowledge of the regulatory mechanisms that occur on the transcriptional level. In embryology everything speaks indeed in favour of a control at this level. Differentiation is believed to be essentially a phenomenon of switch-on and switch-off of certain genes. We have thus to understand how cells communicate and control the commands of such regulatory switches. These are obviously most challenging questions, although I think everybody who has been through the school of simpler systems will appreciate the formidable difficulties foreseeable in this undertaking.

Certainly the understanding of assembly, disassembly and maturation of viruses already teaches us how to understand and how to investigate the corresponding phenomena related to subcellular structures like cytoskeleton and cellular organelles. So many interesting events in the cell are related to the cytoskeleton: the separation of chromosomes during mitosis, the movement of cells, cytoplasmic streaming and transports, among many other cellular functions, are linked to this skeleton. The assembly processes of most of these filamentous structures have been investigated *in vitro* and are found to follow the same basic laws as virus assembly. But *in vivo* so many events occur, which cannot yet become simulated *in vitro*.

The close relationship of the behaviour of subcellular structures with virus multiplication is not very surprising, when remembering that the virus has to use the complete and intact synthesising machinery of the cell, because it only provides information, some sort the blueprint of how it should become built. It is not astonishing either, that by the study of virus multiplication many cellular functions became better understood, like those of the uptake and the excretion of material into and out of the cell.

But all these findings and discoveries on the cellular level have not yet helped to understand more of the form-determining mechanisms governing the forms of plants and animals nor to seize the purpose of the incredible diversity of forms (and colours) encountered. These fundamental features are suitable examples for the necessity of starting with the least complex examples and progressing very slowly to increasing complexities.

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