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Molecular mechanisms controlling protein-protein and protein-nucleic acid interactions as revealed by studies of virus maturation*

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1. Introduction

Many examples are well known where protein biosynthesis is controlled on the level of the transcription of the gene into mRNA. The gene is thus 'switched on or off'. Cellular metabolism, as well as differentiation have been demonstrated to be based on such control mechanisms acting at the transcriptional level. It is suspected that similar controls are exerted on the level of the translation of the messenger into proteins.

On a third level of control the activity of a protein is not determined by absence or presence of this protein, but by a mechanism by which proteins can be shifted from an active to an inactive state. This mechanism has been well investigated in the case of the so-called 'allosterically regulated enzymes'. It is thought that the function of many membrane proteins is also controlled at this level. It seemed important to us to develop methods for exploring this type of mechanisms. The morphogenesis of virus provides an extremely adequate experimental system for such investigations: It is well known that most viruses reproduce according to the following general scheme: After penetration of their nucleic acids into the host cell a first set of genes is expressed under transcriptional control. They concern host virus interactions and a set of enzymes required for the replication of the viral nucleic acid. Only once these first functions have been exerted and nucleic acid starts to become replicated, the second set coding for the late functions becomes expressed. All the late genes - with very rare exceptions - are switched-on at the same time and start to produce proteins steadily at a constant, gene-specific rate. These gene products, which are needed for the assembly and maturation of virus particles, are available from a steadily replenished pool of precursor proteins.

It is well known that, despite this lack of transcriptional control, virus particles undergo quite determined morphogenetic pathways: For many viruses it is known that a precursor particle without nucleic acid is assembled first. It then matures in several steps into the infective virus. One of the maturation steps consists in packaging of nucleic acid. It is important to emphasize here again that the maturation pathway is valid for each individual virus particle, but that different particles within the same cell are not synchronised (figure 1). This is consistent with the observations that it takes 7-15 min to finish a bacteriophage T4 from its constituent amino acids, and that about 5 mature viruses are terminated every minute during 1-2 h. The initiation and maturation of preheads is

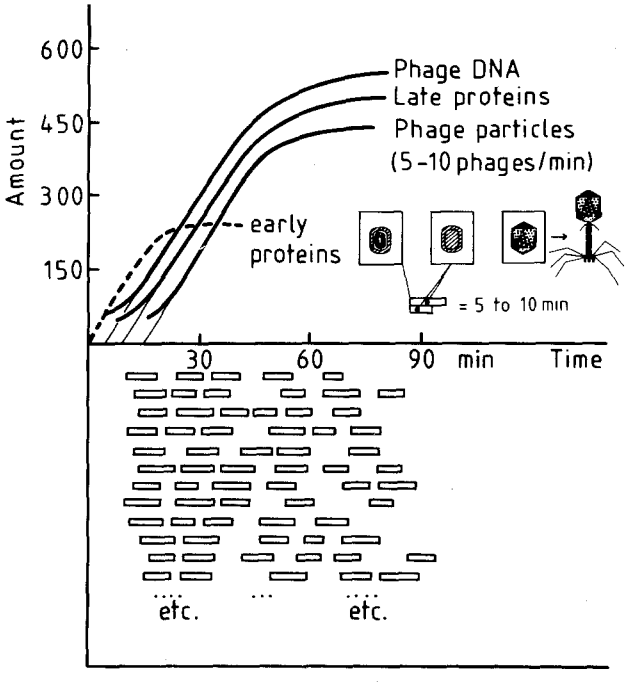

Fig. 1. Asynchrony of virus assembly. Schematic representation of the events governing virus replication. The upper curves represent the synthesis of early and late proteins and of viral DNA in the case of bacteriophage T4 chosen as example. In the lower part the elongated, rectangular boxes represent the maturation path undergone by individual particles. It is given enlarged above and presented with more details on figures 5 and 6.

* The content of this paper covers essentially that of 2 lectures given by the author in China end of November 1979.

thus random in time and absolutely asynchronous. It must therefore be the maturing particle itself which decides which protein-protein or protein-nucleic acid interaction occurs sequentially in time, in order to follow the morphogenetic pathway.

In what follows I will summarize studies made with 2 bacteriophages of *E. coli*, T even (T4 and T2) and λ, in order to investigate and understand some of the mechanisms involved in this sequence of events. We will show in particular that it was possible to demonstrate that some of the morphogenetic steps involved in the maturation of the viral shell are controlled by sequential conformational changes which allow this shell to acquire the ability to interact with additional gene products which are withdrawn from the protein precursor pools.

2. A short presentation of the experimental system

One of the major assets of bacteriophages is their very accessible genetics. Mutants can be produced easily and the assignment of mutants to genes is easily feasible. J. Weigle, L. Siminovitch and I first attempted to use genetics and electron microscopy in problems of phage reproduction on the basis of so-called defective lysogenics of *B. megaterium* (Lwoff and Siminovitch, 1951). Later defective lysogenic strains of λ became available which showed an apparently better potential (Jacob and Wollman, 1956; Arber and Kellenberger-Gujer, 1958). But only the introduction of conditional lethal mutants with phage T4 by Edgar, Epstein, Steinberg and Bernstein in 1960 at the California Institute of Technology provided the real experimental system, which allowed gene-assignment, biochemistry and electron microscopy to become integrated (Epstein et al., 1963). For phage λ such mutants had been introduced already, although published only later (Campbell, 1961); their application to morphogenesis followed (Mount et al., 1968) as well as for several others for which we refer to the reviews mentioned below. Conditional lethal mutants fall into 2 main categories: the so-called 'amber or sus mutants' are selected for giving a normal progeny in one given host but not in another. In this latter, so-called 'non-permissive host', the gene does not give rise to a complete protein but only to a fragment. Indeed, in this non-permissive host no tRNA is available to translate the mutated codon UAG (figure 2). By comparing the bands produced in SDS gel electrophoresis of lysates from infected permissive and non-permissive hosts, it becomes in many cases possible to identify a protein band with the corresponding bacteriophage gene (figure 3). Since in the majority of the cases known the amber fragments lack completely the corresponding function, the morphogenesis is arrested at the precise step in which this gene product should intervene. Instead of the final

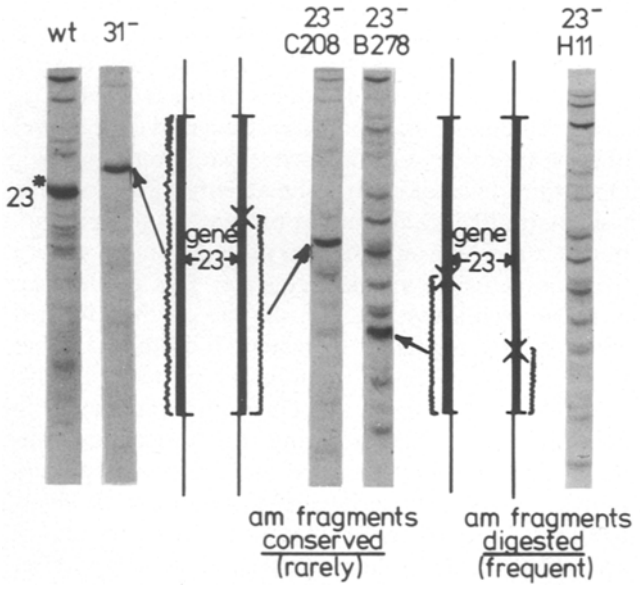

Fig. 3. Gel-electrophoresis of am-fragments. According to the position of the am-mutation in the gene, protein fragments of different lengths are produced. Here we give an example of this colinearity in gene 23 of phage T4 as described by Celis et al., 1973.

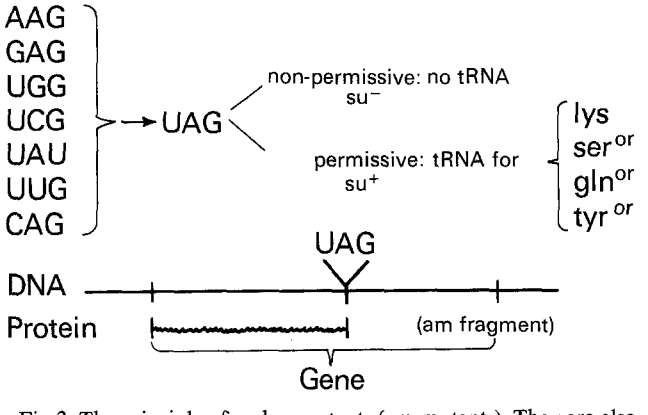

Fig. 2. The principle of amber mutants (am-mutants). They are also called suppressor-sensitive because the nonsense codon is suppressed when an adequate tRNA is available (su⁺-host). Other systems based on other nonsense codons than UAG are known (ochre, opal).

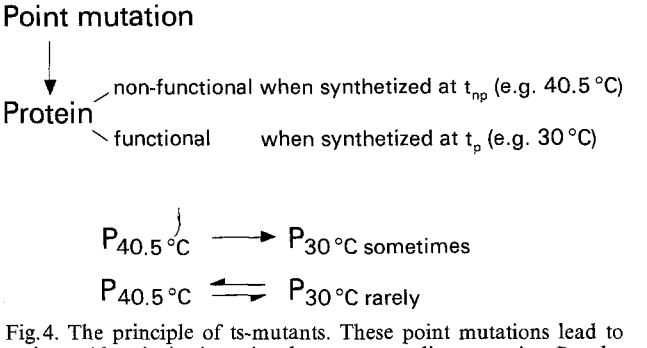

Fig. 4. The principle of ts-mutants. These point mutations lead to amino-acid substitutions in the corresponding protein. By the selection procedure those were selected which produce a non-functional or misfunctional protein at the non-permissive temperature.

virion we observe that in the infected cell the precursor just preceeding a precise step is accumulating. As a complication we have to consider that an abortive pathway might eventually result from this block which leads to abnormal, non-maturable particles.

The temperature sensitive mutants (ts) form the 2nd class of conditional lethal mutants (figure 4). In this case normal particles are produced when the infected cell is cultivated at the so-called 'permissive temperature' while the mutated gene product is inactive or misfunctioning at the non-permissive temperature. Misfunctioning is not rare and ts mutants have thus in every case to be investigated thoroughly before they are used in experiments concerning the morphogenetic pathway. The advantage of ts mutants is that the function of a gene product can frequently become recovered after temperature shift from non-permissive to permissive. In some cases the gene product, the protein, is temperature sensitive itself i.e., it is active at permissive and inactive at non-permissive temperature. In such cases its activity can become controlled by the temperature. In most cases, however, a protein produced at non-permissive temperature stays non-functional even at permissive temperature.

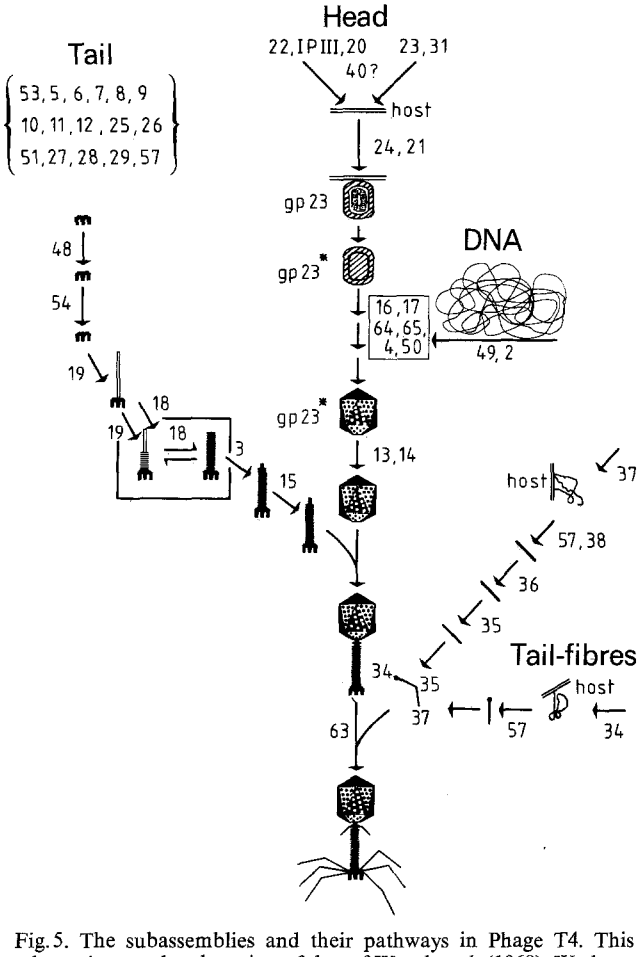

Fig. 5. The subassemblies and their pathways in Phage T4. This scheme is a up-dated version of that of Wood et al. (1968). We have modified particularly the pathway of the head according to recent results shown in more details in figure 6. The first part of the tail assembly has been compressed by graphic reasons. Much more is now known on the base plate assembly through the work of King and his group (Casjens and King, 1975; Wood and King, 1979).

The major problem to become solved experimentally for establishing any morphogenetic pathway is to prove that an observed particle is maturable into an active virus and is thus a true precursor. Such proofs are made by using ts mutants together with temperature shifts, or temporary inhibition by drugs, and by following the flow of radioactive label from one type of particle into another. Particles are investigated and their maturation followed by electron microscopy of thin sections of infected cells, or after artificial rupture, by studying the content of infected cells by ultracentrifugation on sucrose gradients, by electron microscopy and by gel electrophoresis. Very often the proof of a pathway has to be completed by in vitro-experiments. Indeed, precursors can become isolated from mutant-infected cells and many steps of the morphogenesis can thus become performed in the test tube. Pathways of bacteriophages and viruses are summarized in an excellent fashion in 3 recent reviews (Casjens and King, 1975; Butler, 1979; Wood and King, 1979) where a comprehensive set of references are to be found. As an example we show in figure 5 the pathways of bacteriophage T4. From the figure it is clearly visible that the construction of this virus proceeds through 4 different sub-assembly pathways: In one the tail is assembled, in another the head; finished heads and tails join together. Only these attached tails are ready to accept the tail fibres which were assembled independently.

In the following we will only consider the pathway of head maturation. This and related aspects, like the form determination and in vitro assembly, are summarized in more details in a recent review (Kellenberger, 1980), where the reader might find also detailed references.

3. The head maturation pathway of bacteriophage T4

By the methods outlined above, the pathway (figure 6) for the head of bacteriophage T4 was established. Let us remind here that this pathway is followed by any individual particle without being interdependent with that undergone by any other of the many particles produced within the same cell (figure 1). Most of the processes which we now shortly describe are confined to the particle and thus occur in situ. This is particularly striking for the proteolytic events which affect each particle individually, at the exactly propitious moment.

In the so-called 'assembly phase' a prehead consisting of core and shell is assembled out of its protein subunits. The core acts as a scaffold for building the shell. There are indications in vivo and proof in vitro that the core can be assembled independently of the

shell. It is thus not unlikely that cores are first formed and then only become coated by the shell. The prehead (figure 8) contains among many other proteins also gene product 21, which is the zymogen for the T4 prehead protease (T4ppase) (Showe et al., 1976; reviewed by Tsugita et al., 1980). By a still unknown mechanism this zymogen becomes partially proteolytically digested in a cascade of several steps. The resulting T4ppase now acts proteolytically on most of the proteins contained in the prehead. It reduces in particular the molecular weight of the shell subunit (gp23) from 59 kd to 47 kd (gp23*). After this

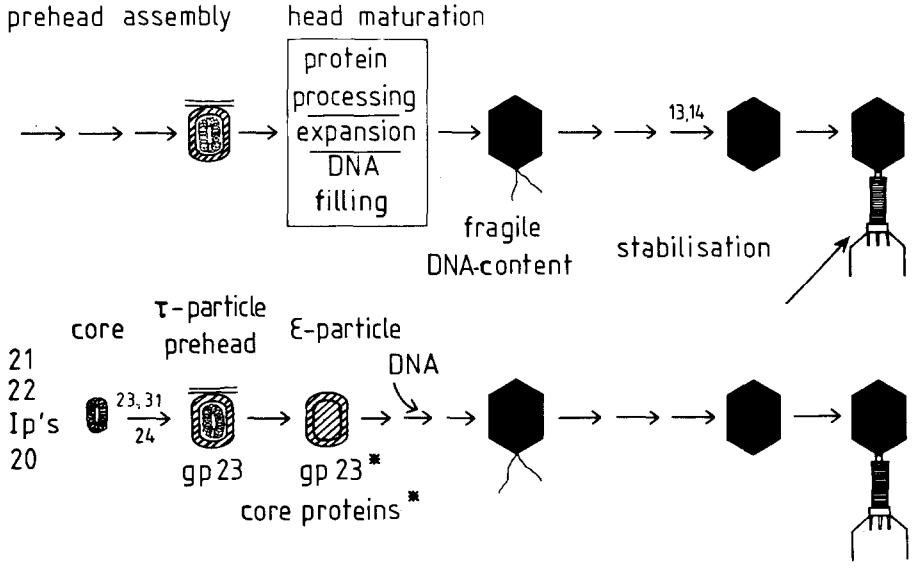

Fig. 6. Pathway of bacteriophage T4 - head maturation. The upper pathway accounts for all the experimental facts reported and which were obtained under various experimental conditions and/or with different mutants. The lower pathway accounts for results obtained under specific conditions (Schaerli and Kellenberger, 1980). It is not excluded, that the order of events summarized in the box might not require a strict sequence in time. The latter might depend on the conditions of growth, like temperature, intracellular concentrations of salts and the like.

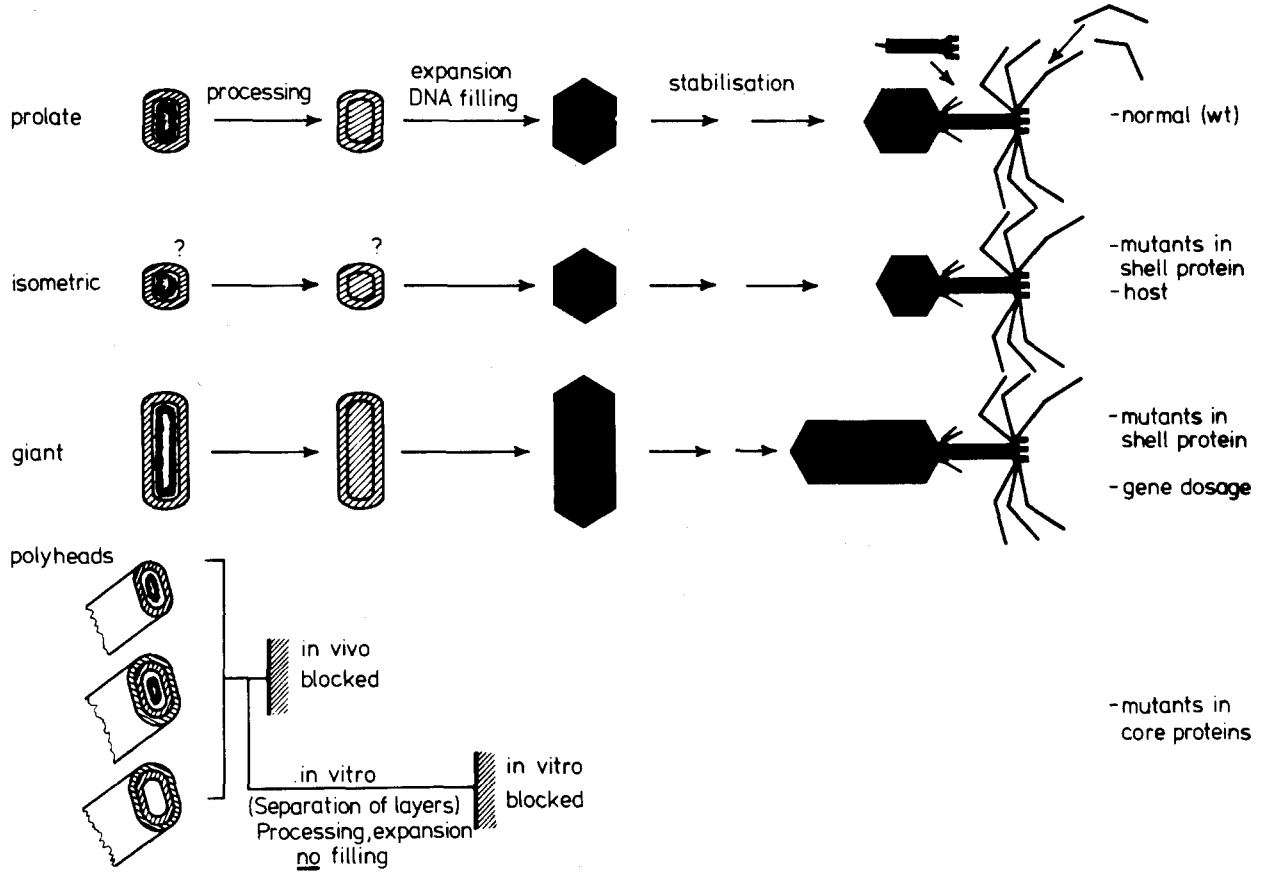

Fig. 7. Polymorphism in bacteriophage T4-heads. The occurrence of form variants is put into relation with the pathway of figure 6. The question mark in the pathway of the isometric head denotes that here it is not yet definitively established that it also goes over isometric preheads. It should be noted that it was not yet possible to assemble isometric preheads in vitro. The basic knowledge on giants is due to work by the groups of Cummings (Cummings and Bolin, 1975) and Doermann (Doermann et al., 1973). For further references and discussion on form determination we refer to Kellenberger (1980).

protein processing has occurred, the result is called ε-particle. This particle becomes now expanded by about 15% in linear dimensions (i.e. its volume increases by about 50%). This expansion occurs without any further addition of subunits to the shell and is thus only due to a conformational change. It is likely that during this expansion the filling with DNA is also initiated. The expanded shell is very much more stable against dissociation, than that of the prehead. It encloses now the DNA which is however still very prone to become lost upon manipulations of the cell as e.g. by disruption (lysis) or chemical fixation for thin sections. By additional steps the particle becomes stabilized and ready to accept the tail.

In this pathway (figure 6) the shells of the prehead, of the intermediate ε-particle and of the head (capsid) are of particular interest for demonstrating conformational changes in correlation with the physiologically defined steps associated with partial proteolysis and with expansion.

Very detailed information can be obtained on 2-dimensional, regular arrays of protein subunits by the procedures of 'image processing' pioneered by Klug et al. (Crowther and Klug, 1975). In these techniques, electron micrographs of such arrays are optically diffracted and the image reconstructed after elimination of the noise represented by statistical density variations in the image. These procedures can also be made mathematically (Fourier-transformation) and calculated with help of a computer.

The above mentioned shells of normal phages and phage precursors are too small for providing sufficient information by these techniques. Fortunately, bacteriophage T4 provides the remedy by one of its particularities: Morphologic variants can be produced as consequence of mutations not only in the major shell protein but mainly in the core proteins. This polymorphism is schematically given in figure 7. For the crystallographic studies we were mainly interested

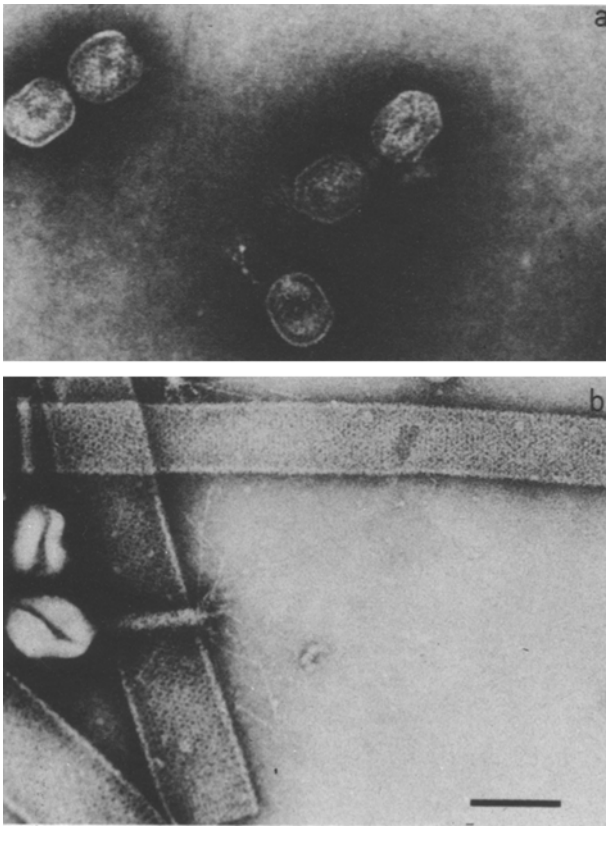

Fig. 8. Phage T4 preheads and its tubular variants. *a* Preheads isolated according to the procedure of Onorato et al. (1978) by R. van Driel and E. Couture-Tosi. *b* 2 empty and flattened down polyheads, the tubular variants of the prehead. A normal phage is also visible and allows comparisons.

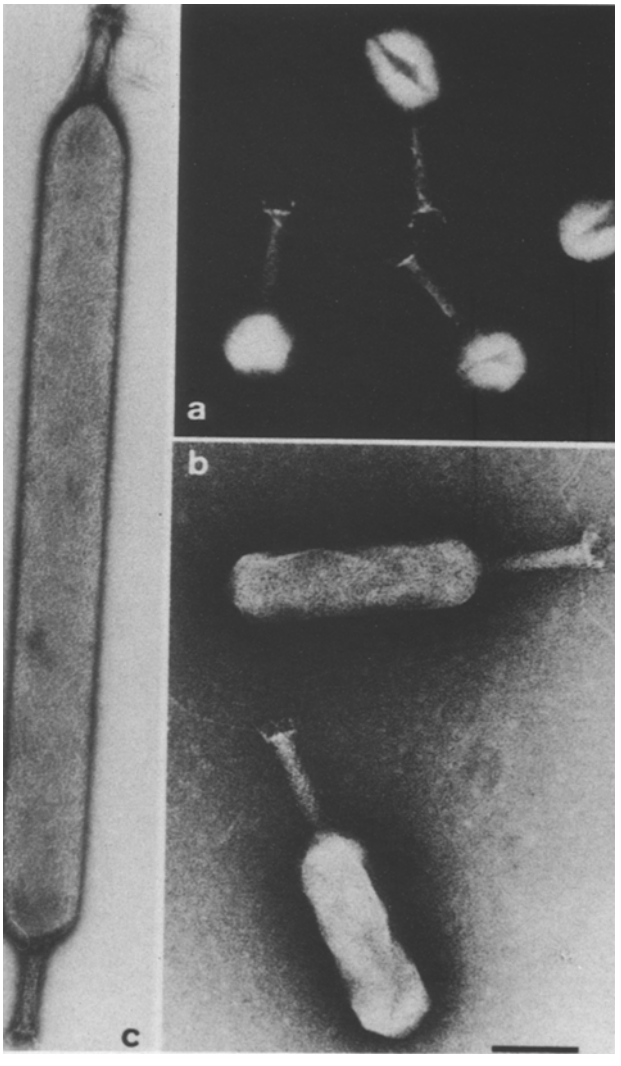

Fig. 9. The size variants of the mature phage T4 heads. On micrographs *a* and *b* by F. Eiserling and *c* by B. ten Heggeler we see *a* normal, prolate and isometric heads; *b* and *c* are giant variants of different lengths. In *b* they contain still their DNA, while in *c* it is lost and thus the shell is flattened. - All these phages are able to inject their DNA content into the host-bacterium. Only the isometric ones contain too little DNA for producing a normal offspring. When infecting with several isometric phages, they complement each other and produce phage, because in phage T4 the DNA contained in the head is cyclically permuted.

in the very elongated forms as represented by the tubular form variants of the prehead - the polyheads (figure 8) - and by the giant phages (figure 9). Polyheads in vivo are abortive, i.e. intracellularly they are never further processed even if T4ppase is present. When isolated they become however a substrate for this enzyme. The giant phage particles follow the normal pathway going through giant preheads and giant ε-particles.

By studying these 2 elongated particles the effects of protein processing and expansion can be correlated by either in vivo or in vitro experiments with the crystallographic parameters of the surface lattice of these shells.

4. The conformational changes of the shell accompanying the maturation of prehead into head

The results of experiments, as those outlined above (for references, see Kellenberger, 1980, and figure 12), can be summarized as follows: in figure 11 we give the surface lattices of the prehead, of the ε-particle, and of the capsid (figure 10). Micrographs of negatively stained, empty particles were processed either by optical diffraction and filtering or/and by computer. The white dots can be thought as being the part of a protein subunit (gp23) which emerges out of the sea of negative stain. One has obviously to assume that the different subunits are in close contact even without this contact areas being visible because we know from the mechanical properties that the subunits are strongly connected together by chemical interactions. Since we know also that no covalent bonds are involved, we have to assume rather large areas of contacts, involving high numbers of weak interactions. On these lattices one can now determine some parameters as the unit cell length, i.e. the distance between the capsomers formed by 6 identical subunits and the orientation angle of a capsomer in respect to a lattice line. These parameters are given under the reconstructed micrographs in figure 11. After the proteins of the prehead have been processed, the lattice constant is not changed, but the orientation angle of the capsomer becomes significantly different. We thus have already a conformational change which is in the order of magnitude of some 7Å. A profound change of the lattice occurs after expansion. The lattice constant is now substantially increased and the orientation angle is also completely different. It is interesting to note that now the shell is extremely stable and the subunit interactions have become very strong as mentioned already.

The data presented until here demonstrate unambiguously a change of quarternary structure. They,

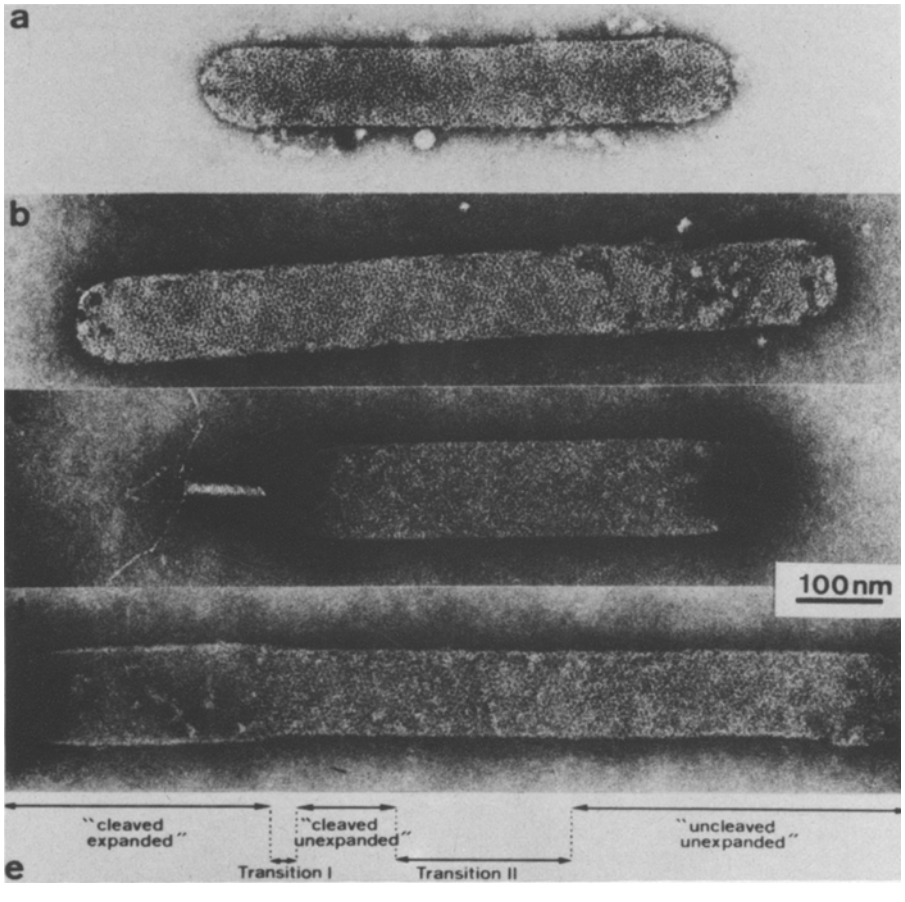

Fig. 10. Giant phage T4-related particles at different stages of maturation. On these micrographs from J. Carrascosa we show the negatively stained shells of canavanine-induced giant variants of *a* preheads, *b* ε-particles and *c* capsids. The crystallographic analysis of the surface lattices of such particles are given in figure 11. In *e* we show one of the very few particles of which the shell is subdivided into three regions corresponding to the three lattice conformations, depicted in figure 11. They are separated by regions of transition.

however, are not sufficient to decide between a simple rearrangement of rigid subunits or a tertiary structure change of the protein subunits themselves. This question is discussed below, and in more details elsewhere. The morphogenetic pathway stops here for bacteriophage T2 and the lattice shown in figure 11c is the final structure of the capsid of this virus. In phage T4 we face an additional event. Japanese workers had shown that T4 has additional minor proteins on its shell (Ishii and Yanagida, 1975). For these minor proteins it was possible to show that they can interact with the shell only after it is expanded (Ishii et al., 1978; Carrascosa, 1978). As visible in figure 12, these minor proteins *hoc* and *soc* interact with the expanded

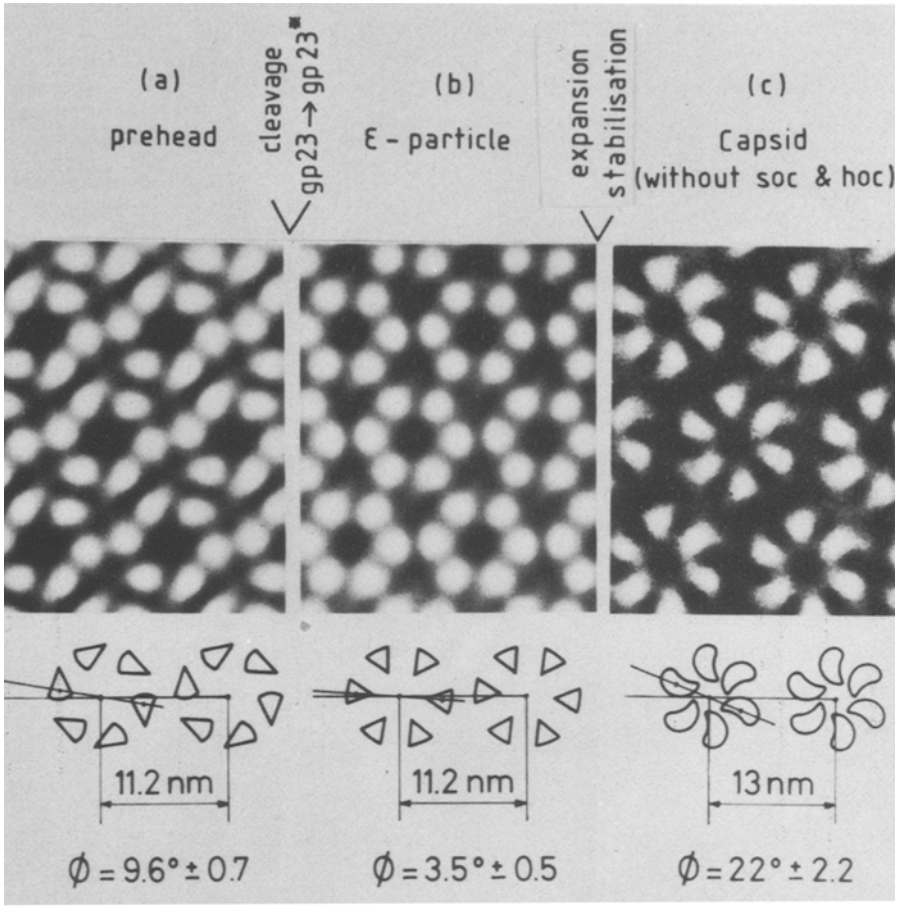

Fig. 11. The lattice transformations correlated with the maturation of the phage T4 head shell. These image-processed micrographs from A. Steven and J. Carrascosa, represent the negatively stained lattices (a) of the prehead, (b) of the ε-particle and (c) of the final, stable capsid before *soc* and *hoc* are bound to it. The crystallographic analysis was done on particles as those represented in figure 10. Below the micrographs, a schematic drawing of the hexamerically arranged subunits is given, with the measured parameters. Between (a) and (b) the shell protein was proteolytically pared away by 20% by the action of T4ppase, and between (b) and (c) expansion occurred. (For references see figure 12.)

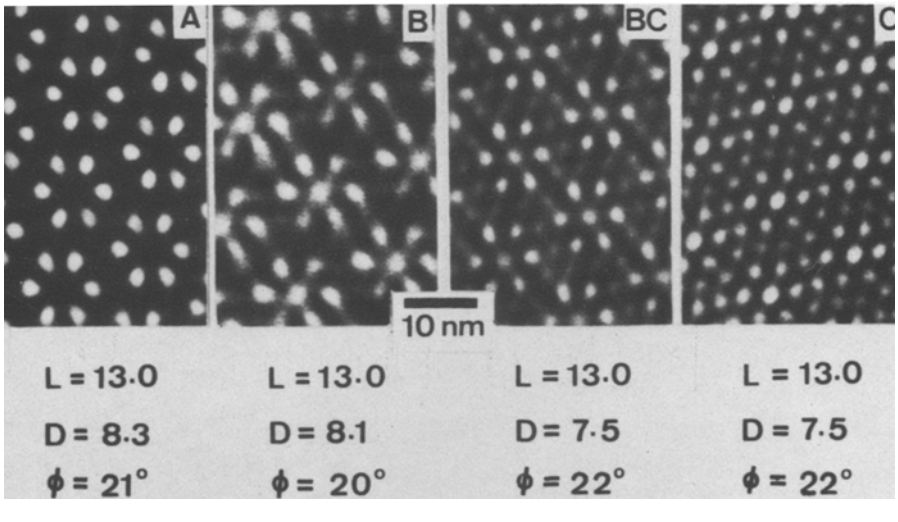

Fig. 12. The addition of the minor proteins *soc* and *hoc* to the T4-capsid. These processed micrographs are due to efforts of U. Aebi, J. Kistler (then graduate students), B. ten Heggeler and A.C. Steven with the advice of M. Showe and R.P. Smith. To the expanded, stabilized surface lattice *A* (which is the same as in figure 8c), the minor proteins *hoc* and *soc* are now able to bind. *B* shows *hoc* bound in the center of the capsomere. On *BC* and *C* increasing amounts of *soc* are bound and surround the capsomers.

Figures 11 and 12 summarize results spread in the following publications: Laemmli et al., 1976; and from our laboratory: Aebi et al., 1977b and c; Bijlenga et al., 1976; Steven et al., 1976a and b; Steven and Carrascosa, 1979.

shell by becoming fixed either in the center of the capsomer *(hoc)* or symmetrically surrounding it *(soc)*. This experiment clearly demonstrates that the sites necessary for this interaction became available only after the last step of the shell maturation, the expansion, has occurred. The addition of these minor proteins further increases slightly the stability of the shell. This increase is, however, not vital because, as we have seen above, a variant of this bacteriophage, the T2, is perfectly viable without them, as do mutants of T4 which lack *soc* and *hoc*. Bacteriophage λ, however, presents a case in which the added protein is absolutely indispensible for producing a viable virus.

5. The lattice transformations accompanying the maturation of the shell in bacteriophage λ

In this bacteriophage (Hohn and Katsura, 1977) the head maturation does not involve cleavage of the major shell protein. It, however, shows expansion as in the case of phage T4. The shell of the prehead is made of the protein produced by gene E (gpE). After expansion, gpD can bind to it. Only with this additional protein added in amounts equal to gpE, the shell is enough stable to produce a viable particle containing a full complement of DNA. A very fragile particle, which is infective, can become assembled when a reduced amount of DNA becomes packaged into the head.

The shell maturation in this phage was simulated in vitro by producing tubular forms, so-called 'polyheads', which were expanded by action of urea and to which then gpD became bound. These lattices were also studied by the crystallographic techniques mentioned above (Wurtz et al., 1976). Because of a particular symmetry of these tubular forms the produced images are less faithfull for details than those of phage T4. Nevertheless, a significant, pronounced, conformational change is quite obvious from figure 13. Again the additional gpD can interact only with the expanded lattice of gpE.

6. Conclusions and discussion

From the results reported above for phage T4 and phage λ it becomes evident that a profound conformational change, the expansion, is necessary for revealing interaction sites necessary for binding 1 or 2 additional proteins. These additional gene products were available in the cell, in a precursor pool, ready to be put into action, i.e. to be actuated, by the particle. It is thus the particle which decides about the precise moment at which it needs this interaction with an additional protein. The interaction is not controlled by the sudden appearance of this protein or by its activation. It is a fully passive partner, while the precursor particle is the active one, which becomes activated by a conformational change. Let us now compare the situation with that of allosteric enzymes, which we had mentioned in the introduction. Here the

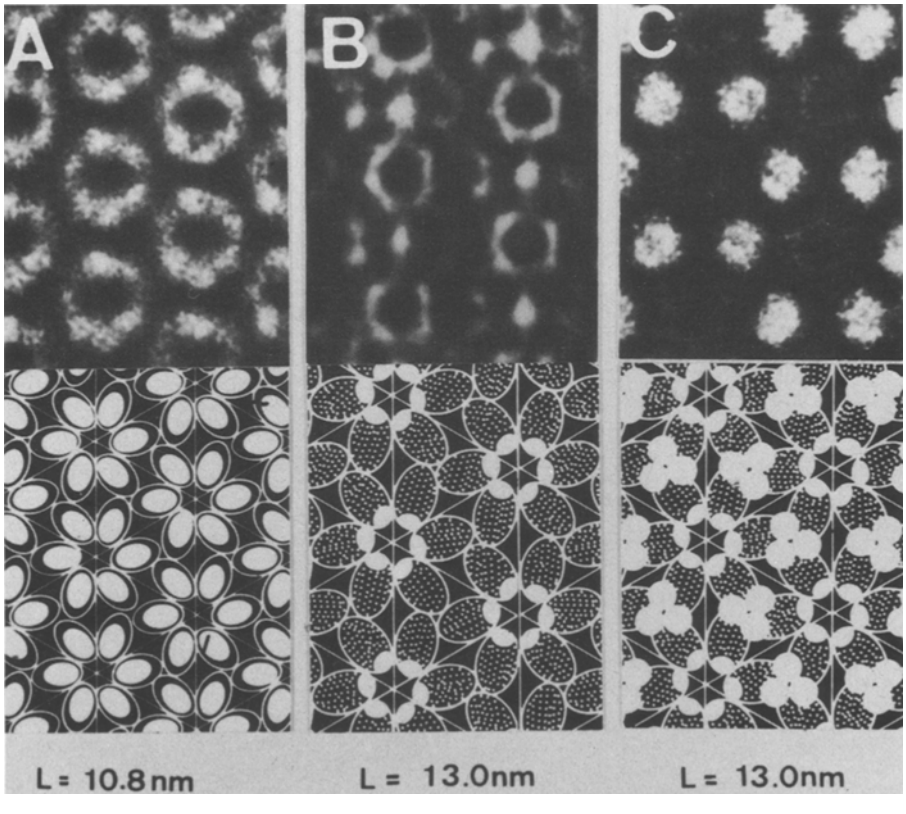

Fig. 13. Lattice transformations during maturation of the shell of bacteriophage λ. These processed micrographs from M. Wurtz were made in collaboration with Th. and B. Hohn. They show the negatively stained lattices *A* of the prehead, constituted only of gpE, *B* after expansion of this lattice, when it has generated binding sites for gpD; on *C* gpD is shown bound to the lattice. Below the processed micrographs the subunit-arrangement is shown schematically. The stoichiometry of gpD to gpE is 1. The crystallographic analysis was done on in vitro reassembled tubular variants of the prehead. The bright spots on *c* are trimers of gpD, which protrude from the surface. For references see text.

enzyme becomes activated by an induced conformational change, so as to interact with the substrate. The substrate is here the passive partner which is actuated. For a complete understanding of this mechanism of actuation of proteins, it will be important to definitively establish that the observed conformational changes are not only based on the rearrangement of rigid proteins by which hidden interaction sites become available, but that new sites are generated on the protein subunits by a tertiary structure change. In T4 freeze-drying (Kistler et al., 1978) and labelling by the Fab fragment of specific antibodies (Aebi et al., 1977a) has already given evidence for the latter alternative. We discuss elsewhere (Kellenberger, 1980) the additional logical arguments in favour of tertiary structure changes, which are the consequences of the specificity of the interaction sites and of the enormous increase of their bonding strength as correllary of the maturation of the shell.

It is most likely that this new control mechanism is also responsible for many other steps in the maturation of the bacteriophage head. It explains easily the initiation of the proteolytic cascade of the zymogen which leads to the T4ppase. If it becomes true that the core of the prehead is assembled prior to the addition of its shell, then we have also to find an explanation why the shell protein interacts with the core proteins only once the core has its correct form. Again, induced conformational changes can easily explain the generation of the newly required interaction sites.

This mechanism also easily explains the many steps of the beautifully investigated morphogenetic pathways of the tails of phages T4 and λ (Casjens and King, 1975; Wood and King, 1979; Hohn and Katsura, 1977). Unfortunately, it is much more difficult to demonstrate conformational changes on the tail assembly pathway than on that of giant shells.

The principle of sequentially induced conformational changes as a basis for sequential protein-protein and protein-nucleic acid interactions, was proposed earlier (Kellenberger, 1966; 1972). It is rewarding to see that this postulated mechanism does really occur. It seems to us obvious that similar mechanisms are likely to be involved not only in the assembly of other viruses, but also in that of cellular organelles and, as already mentioned, in the control of the assembly and of the functions of biological membranes, like active transport, signal transmission, proton pumping, to name only a few. It was relatively easy to develop adequate technical approaches with bacteriophages while it would have been difficult to do so on more complex cellular systems. We very much hope that by this contribution we might encourage the extension and adaptation of this approach to more complex systems in which conformational differences are suspected. Since it appears also that the techniques of electron microscopy may become further improved (for a discussion see Kellenberger, 1978) an important facet of molecular biology might thus become more accessible to research.

Acknowledgments. Many of the experimental results, particularly those presented in the figures of this summary were achieved at one time or another when the authors (professors on sabbatical leave, postdoctoral fellows, graduate students and technicians) worked in my laboratory within research projects granted to me by the Swiss National Science Foundation. A.H. Doermann with L. Boehner isolated painfully mutants producing morphological variants during their stay in my laboratory then at the Basel Institute for Immunology. There they started work with F. Eiserling with whom they continued collaboration after they had all left (Doermann et al., 1973). With their contribution they laid the basis for the investigation of the following colleagues and collaborators who were with me at their times: A.C. Steven, J. Carrascosa, U. Aebi, J. Kistler, B. ten Heggeler, who used the procedures of information processing set up by P.R. Smith with the help of U. Aebi, A.C. Steven and with contributions by E. Boy de la Tour and M. Wurtz. They collaborated closely with my colleague M.K. Showe and L. Onorato, who at that time had just discovered and characterized the T4ppase. During their stay in my laboratories Thomas and Barbara Hohn with collaborators had formed a highly efficient group for investigating the morphogenesis of phage λ. To all of them, who had to leave the laboratory by virtue of the structure of our institution, go my sincere thanks for having accepted to work and contribute creatively to these problems which, at that time, were not as attractive as they look now in retrospective.

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SPECIALIA

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^{13}C NMR-study of flexibilide, an anti-inflammatory agent from a soft cora[1]

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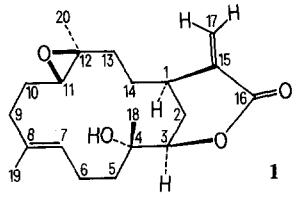
Summary. The use of ^{13}C spin-lattice relaxation measurements as a probe of structure in solution is illustrated for flexibilide. Analysis of quaternary carbon relaxation behaviour indicates that the structure in solution differs from the crystal structure. The effects of lanthanide shift and relaxation reagents on ^{13}C spectral parameters are also reported.

Flexibilide (**1**), a cembranoid lactone isolated from the soft coral *Sinularia flexibilis*[3,4], exhibits anti-inflammatory and anti-arthritic activity in the rat[5]. We have examined the structure of **1** in solution with the use of ^{13}C NMR spin-lattice relaxation measurements, placing particular emphasis on the analysis of quaternary carbon relaxation behaviour as a structural probe.

Materials and methods. Flexibilide was isolated from *S. flexibilis* as described previously[3]. Yb(fod)$_3^6$ and Gd(fod)$_3$ were obtained from Norell Chemical Co., Landisville, NJ, and Eu(fod)$_3$ and Pr(fod)$_3$ from Pierce Chemical Co., Rockford, Ill. The solvents $CDCl_3$, $(CD_3)_2SO$ and D_2O were all spectroscopic grade (> 99% deuterated). All materials were used as received.

Natural-abundance ^{13}C NMR-spectra were obtained at 15.04 MHz on a Jeol FX-60 spectrometer operating in the pulsed Fourier transform mode, with on-resonance noise-modulated proton decoupling and 10 mm OD spinning sample tubes. Probe temperature was 32 ± 1 °C. All spectra were accumulated in 8192 time-domain addresses, and processed with 0.7–1.2 Hz exponential broadening. Samples were degassed by bubbling nitrogen through the solutions for at least 1 min, and the NMR-tubes were capped and sealed with Parafilm. Spin-lattice relaxation times (T_1) were measured by the inversion-recovery method[7]. The 90° pulse width was 17 μsec.

Results and discussion. The figure shows the ^{13}C-spectrum of **1** in $CDCl_3$. The table summarizes ^{13}C chemical shifts, spin-lattice relaxation times (T_1) and integrated intensities for the 20 carbons of **1** in $CDCl_3$ and DMSO-d_6. Resonance assignments are based on chemical shifts[8], multiplicities in single-frequency off-resonance proton-decoupled spectra, and T_1-values. In both solvents all carbons (protonated and quaternary) have nuclear Overhauser enhancements (NOE) of 3.0 ± 0.3, indicating that their spin-lattice relaxation is dominated by ^{13}C-^{1}H dipolar interactions[9,10]. Furthermore, in each solvent the T_1-values of the methine carbons are identical within experimental error, and are twice as long as the values for the methylene carbons. This indicates[9,10] that **1** undergoes essentially isotropic reorientation in $CDCl_3$ and DMSO-d_6, and that any internal motions in the molecule are slow relative to the rate of overall molecular tumbling. The exceptions are the methyl groups, which would have T_1-values of 0.33 and 0.13 sec in $CDCl_3$ and DMSO-d_6, respectively, in the absence of internal motion. The data in the table indicate that all 3 methyl groups



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