FROM MACROMOLECULES TO BIOLOGICAL ASSEMBLIES

Nobel lecture, 8 December, 1982

by

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Within a living cell there go on a large number and variety of biochemical processes, almost all of which involve, or are controlled by, large molecules, the main examples of which are proteins and nucleic acids. These macromolecules do not of course function in isolation but they often interact to form ordered aggregates or macromolecular complexes, sometimes so distinctive in form and function as to deserve the name of organelle. It is in such biological assemblies that the properties of individual macromolecules are often expressed in a cell. It is on some of these assemblies on which I have worked for over 25 years and which form the subject of my lecture today.

The aim of our field of structural molecular biology is to describe the biological machinery, in molecular, i.e. chemical, detail. The beginnings of this field were marked just over 20 years ago in 1962 when Max Perutz and John Kendrew received the Nobel prize for the first solution of the structure of proteins. In the same year Francis Crick, James Watson, and Maurice Wilkins were likewise honoured for elucidating the structure of the double helix of DNA. In his Nobel lecture Perutz recalled how 40 years earlier, in 1922, Sir Lawrence Bragg, whose pupil he had been, came here to thank the Academy for the Nobel prize awarded to himself and his father, Sir William, for having founded the new science of X-ray crystallography, by which the atomic structure of simple compounds and small molecules could be unravelled. These men have not only been my predecessors, but some of them have been something like scientific elder brothers to me, and I feel very proud that it should now be my turn to have this supreme honour bestowed upon me. For the main subjects of my work have been both nucleic acids and proteins, the interactions between them, and the development of methods necessary to study the large macromolecular complexes arising from these interactions.

In seeking to understand how proteins and nucleic acids interact, one has to begin with a particular problem, and I can claim no credit for the choice of my first subject, tobacco mosaic virus. It was the late Rosalind Franklin who introduced me to the study of viruses and whom I was lucky to meet when I joined J.D. Bernal's department in London in 1954. She had just switched from studying DNA to tobacco mosaic virus, X-ray studies of which had been begun

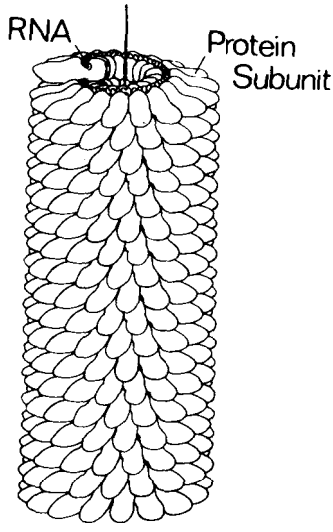


Fig. 1. Diagram summarizing the results of the first stage of structure analysis of tobacco mosaic virus (71). There are three nucleotides per protein subunit and $16^{1}/_{3}$ subunits per turn of the helix. Only about one-sixth of the length of a complete particle is shown.

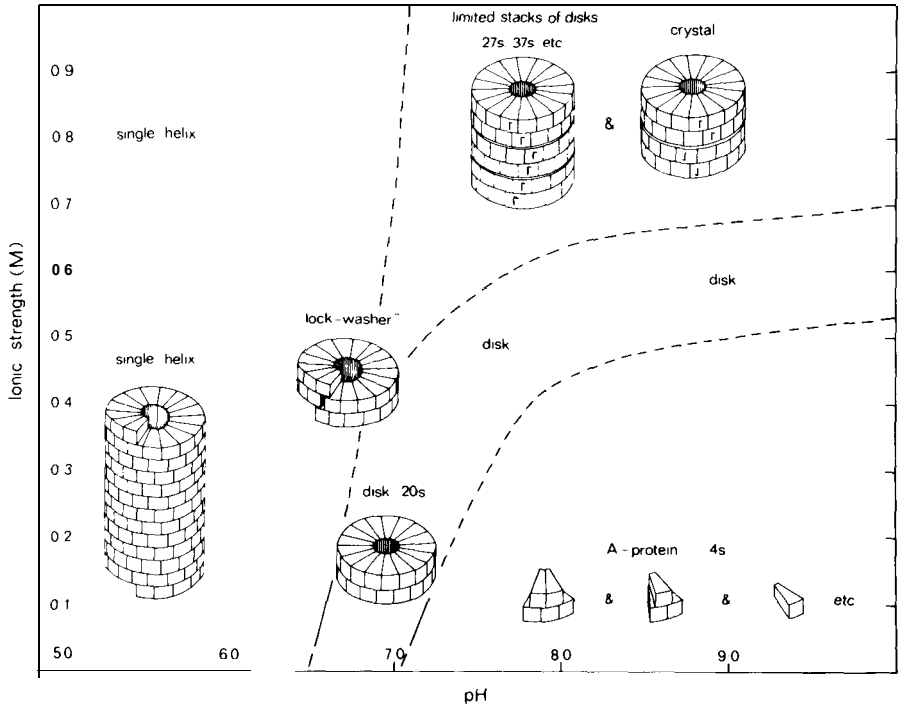


Fig. 2. Diagram showing the ranges over which particular forms of TMV protein participate significantly in the equilibrium (17). This is not a conventional phase diagram: a boundary is drawn where a larger species becomes detectable and does not imply that the smaller species disappears sharply. The "lock washer" indicated on the boundary between the 20S disk and the helix is not well defined and represents a metastable transitory state observed when disks are converted to helices by abrupt lowering of the pH.

by Bernal in 1936. It was Rosalind Franklin who set me the example of tackling large and difficult problems. Had her life not been cut tragically short, she might well have stood in this place on an earlier occasion.

TOBACCO MOSAIC VIRUS

Tobacco mosaic virus (TMV) is a simple virus consisting only of a single type of protein molecule and of RNA, the carrier of the genetic information. Its simple rod shape results from its design, namely a regular helical array of these protein molecules, or subunits, in which is embedded a single molecule of RNA. This general picture was already complete by 1958 when Rosalind Franklin died (Fig. 1). It is clear that the protein ultimately determines the architecture of the virus, an arrangement of $16^1/_3$ subunits per turn of a rather flat helix with adjacent turns in contact. The RNA is intercalated between these turns with 3 nucleotide residues per protein subunit and is situated at a radial distance of 40 Å from the central axis and is therefore isolated from the outside world by the coat protein. The geometry of the protein arrangement forces the RNA backbone into a moderately extended single-strand configuration. Running up the central axis of the virus particle is a cylindrical hole of diameter 40 Å, which we then thought to be a trivial consequence of the protein packing, but which later turned out to figure prominently in the story of the assembly.

At first sight, the growth of a helical structure like that of TMV presents no problem of comprehension. Each protein subunit makes identical contacts with its neighbours so that the bonding between them repeats over and over again. Subunits can have a precise built in geometry so that they can assemble themselves like steps in a spiral staircase in a unique way. Subunits would simply add one or a few at a time onto the step at the end of a growing helix, entrapping the RNA that would protrude there and generating a new step, and so on. It was in retrospect thus not too surprising when the classic experiments of Fraenkel-Conrat and Williams in 1955 (6) demonstrated that TMV could be reassembled from its isolated protein and nucleic acid components. They showed that, upon simple remixing, infectious virus particles were formed that were structurally indistinguishable from the original virus. Thus all the information necessary to assemble the particle must be contained in its components, that is, the virus "self assembles". Later experiments ('7) showed that the reassembly was fairly specific for the viral RNA, occuring most readily with the RNA homologous to the coat protein.

All this was very satisfactory but there were yet some features which gave cause for doubt. First, other experiments (8) showed that foreign RNAs could be incorporated into virus-like rods and these cast doubt on the belief that specificity *in vivo* was actually achieved during the assembly itself. Another feature about the reassembly that suggested that there were still missing elements in the story was its slow rate. Times of 8 to 24 hours were required to give maximum yields of assembled particles. This seemed to us rather slow for the assembly of a virus *in vivo,* since the nucleic acid is fully protected only on

completion. These doubts, however, lay in the future and before we come to their resolution, I return to the structural analysis of the virus and the virus protein.

X-ray analysis of TM V: the protein disc

After Franklin's death, Holmes and I continued the X-ray analysis of the virus. Specimens for X-ray work can be prepared in the form of gels in which the particles are oriented parallel to each other, but randomly rotated about their own axes. These gels give good X-ray diffraction patterns but because of their nature the three-dimensional X-ray information is scrambled into two dimensions. Unscrambling these data to reconstruct the 3-dimensional structure has proved to be major undertaking, and it was only in 1965 that Holmes and I obtained the first 3-dimensional Fourier maps to a resolution of about 12 Å. In fact, only recently has the analysis by Holmes and his colleagues in Heidelberg (where he moved in 1968) reached a resolution approaching 4 Å in the best regions of the electron density map, but falling off significantly in other parts (9). At this resolution it is not possible to identify individual amino-acid residues with any certainty and ambiguities are too great to build unique atomic models. However, the map, taken together with the detailed map of the subunit we obtained in Cambridge (see below) yields a considerable amount of information about the nature of the contacts with RNA (10).

These difficulties in the X-ray analysis of the virus were foreseen, and by the early 1960's I came to realize that the way around this difficulty was to try to crystallize the isolated protein subunit of the virus, solve its structure by X-ray diffraction and then try to relate this to the virus structure solved to low resolution. We therefore began to try to crystallize the protein monomer. In order to frustrate the natural tendency of the protein to aggregate into a helix, Leberman introduced various chemical modifications in the hope of blocking the normal contact sites, but none of these modified proteins crystallised. The second approach was to try to crystallize small aggregates of the unmodified protein subunits. It had been known for some time, particularly from the work of Schramm and Zillig (11), that the protein on its own, free of RNA, can aggregate into a number of distinct forms, besides that of the helix. I chose conditions under which the protein appeared to be mainly aggregated in a form with a sedimentation constant of about 4S, identified by Caspar as a trimer (12). We obtained crystals almost immediately but we found (13) them to contain not the small aggregate hoped for, but a large one, corresponding to an aggregate with a sedimentation constant of 20S. The X-ray analysis showed that this was built from two juxtaposed layers, or rings, of 17 subunits each and we named this form the two-layer disc (Fig. 3 and 4). Our inital dismay in being faced with such a large structure, of molecular weight 600,000, was tempered by the fact that the geometry of the disc was clearly related to that of the virus particle. The cylindrical rings contained 17 subunits each compared with $16^1/_3$ units per turn of the virus helix, so that the lateral bonding within the discs was therefore likely to be closely related to that in the virus. We also

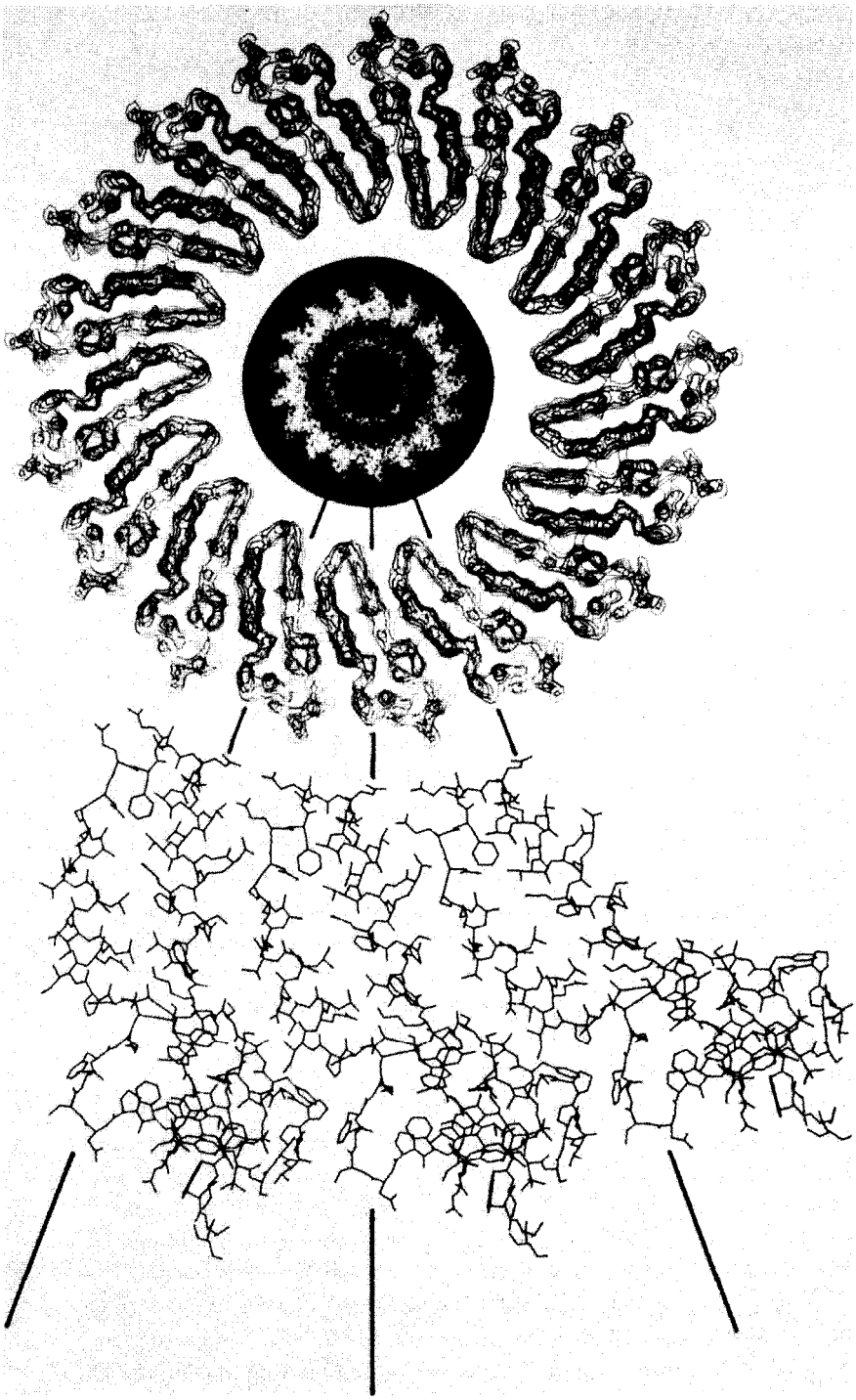


Fig. 3. The disk viewed from above at successive stages of resolution. From the centre outward there follow (i) a rotationally filtered electron microscope image at about 25 Å resolution (72); (ii) a slice through the 5 Å electron density map of the disk obtained by X-ray analysis, showing rod-like α-helices (26) and (iii) part of the atomic model built from the 2.8 Å map (Bloomer et al., ref. 15).

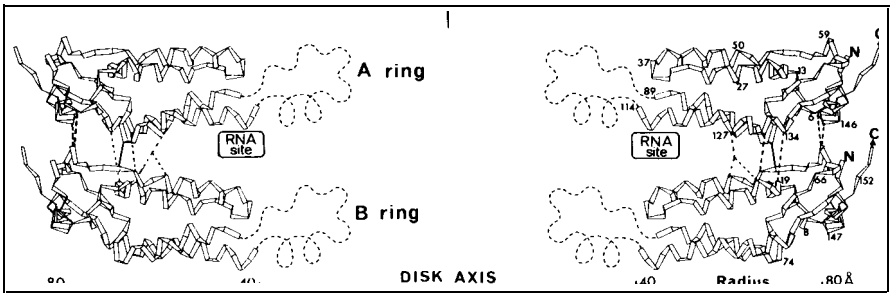


Fig. 4. Section through a disk along its axis reconstructed from the results of X-ray analysis to a resolution of 2.8 Å (15). The ribbons show the path of the polypeptide chain of the protein subunits. Subunits of the two rings can be seen touching over a small area toward the outside of the disk but opening up into the "jaws" toward the centre. The dashed lines at low radius indicate schematically the mobile portion of the protein in the disk, extending in from near the RYA binding site to the edge of the central hole

showed, by analysing electron micrographs, that the disc was polar, i.e. that its two rings faced in the same direction as do successive turns of the virus helix.

This was the first very large structure ever to be tackled in detail by X-ray analysis and it took about a dozen years to carry through the analysis to high resolution. The formidable technical problems were overcome only after the development in our laboratory of more powerful X-ray tubes and of special apparatus (cameras, computer-linked densitometers) for data collection from a structure of this magnitude. (In fact we had begun building better X-ray tubes in London to use on weakly diffracting objects like viruses). The 17-fold rotational symmetry of the disc also gives rise to redundant information in the X-ray data, which was exploited in the final analysis (14), to improve and extend the resolution of a map based originally on only one heavy atom derivative. The map at 2.8 Å resolution (15) has been interpreted in terms of a detailed atomic model for the protein (Figs. 3 and 4), although the individual interactions upon RNA binding have yet to be deduced.

Protein polymorphism

These results on the structure of the disc which showed that it was fairly closely related to the virus helix made me wonder whether the disc aggregate might not be fulfilling some vital biological role. It had been easy to dismiss it as perhaps an adventitious aggregate of a sticky protein or a storage form. The polymorphism of TMV protein was first considered in some detail by Caspar in 1963 (12) who foresaw that some of the aggregation states might give insight into the way the protein functions. Quantitative studies of aggregation started by Lauffer in the 1950's (16) concentrated upon a rather narrow range of conditions, the main interest being in understanding the forces driving the aggregation (these are largely entropic). Because of the scattered nature of the earlier observations, Durham, Finch and I began a systematic survey of the aggregation states as a result of which the broad outline became clear (17, 18). The results can be summarised as a phase diagram (Fig. 2).

At low or acid pH, the protein alone will form helices of indefinite lengths that are structurally very similar to the virus except for the lack of the RNA. Above neutrality the protein tends to exist as a mixture of smaller aggregates from about trimer upwards, in rapid equilibrium with each other, commonly referred to as A-protein. Near pH 7 and at about room temperature the dominant form present is the disc which is in a relatively slow equilibrium with the A-form in the ratio of about 4:1. The dominant factor controlling the state of aggregation of the coat protein is thus the pH. The control is mediated through groups, probably carboxylic acid residues, as identified by Caspar (12), that bind protons abnormally in the helical state, but not in the disc or A-form. Thus the helical structure can be stabilised either in the virus by the interaction of the RNA with the protein, or, in the case of the free protein, by protonating the acid groups. These groups thus act as a "negative switch", ensuring that under physiological conditions the helix is not formed, and thus that enough protein in the form of discs or A-protein is available to interact with the RNA during virus assembly.

A role for the disc

The disc aggregate of the protein therefore has a number of significant properties. It is not only closely related to the virus helix, but also is the dominant form of the protein under "physiological" conditions; moreover, disc forms had also been observed for other helical viruses. These strengthened my conviction that the disc form was not adventitious but might play a significant role in the assembly of the virus. What could this role be?

Assembly of any large aggregate of identical units such as a crystal can be considered from the physical point of view in two stages: first nucleation and then the subsequent growth, or, in more biochemical language, as initiation and subsequent elongation. The process of nucleation - or, crudely, getting started - is frequently more difficult than the growth. Thus, a simple mode of initiation in which the free RNA interacts with individual protein subunits does pose problems in getting started. At least 17 separate subunits would have to bind to the flexible RNA molecule before the assembling linear structure could close round on itself to form the first turn of the virus helix. This difficulty could be avoided if a preformed disc were to serve as a jig upon which the first few turns of the viral helix could assemble to reach sufficient size to be stable. This mode of nucleation of helix assembly could also furnish a mechanism for the recognition by the protein of its homologous RNA. The surface of the disc presents a set of 51 (= 17 3) nucleotide binding sites which could interact with a special long run of bases, resulting in an amplified discrimination that might not be possible with a few nucleotides. It thus seemed that the disc could solve both the physical and biological requirements for initiating virus growth and conferring specificity on the interaction. This hypothesis is illustrated in Fig. 5. It turned out that all the details in this diagram are wrong, but yet the spirit is correct. As A.N. Whitehead once observed, it is more important that an idea should be fruitful than it should be true.

This proposed mechanism of nucleation required that the disc be able to

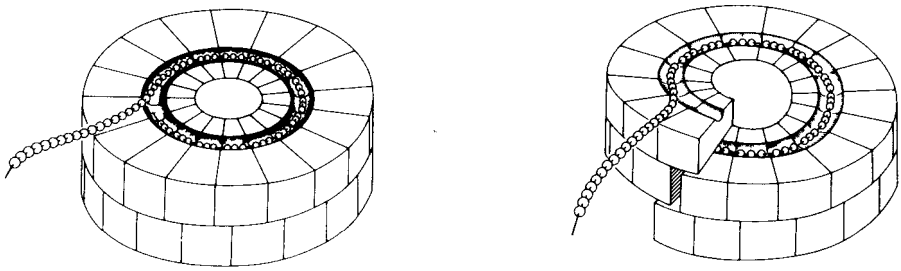


Fig. 5. The role of the disk as originally conceived: the specific recognition of a special (terminal) sequence of TMV-RNA initiates conversion of the disc form of the protein into two turns of helix, (See Fig. 7, for the mechanism finally established.)

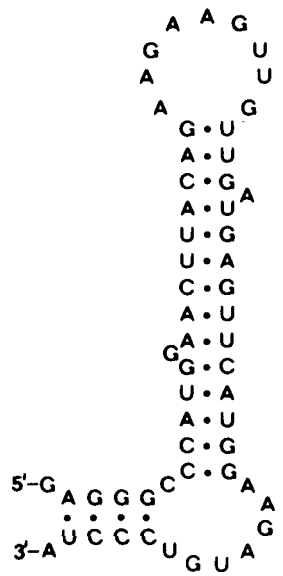
dislocate into a two-turn helix to form the beginning of the growing nucleoprotein rod. To test this, we carried out a very simple experiment, the pH drop experiment (19). This showed that an abrupt lowering of the pH would convert discs directly, within seconds, into short helices - or lockwashers (Fig. 2), which stack on each other to give longer nicked helices, which in due course anneal to give more perfect hehces. This conversion is an *in situ* one, not requiring dissociation and then reassociation into a different form. The success of this experiment encouraged us to proceed to experiments with RNA itself, the natural "substrate" of the virus protein.

The first reconstitution experiments carried out by Butler and myself proved to be dramatic (20). When a mixture was made at pH 7 of the viral RNA and a disc preparation, complete virus particles were formed within 10 to 15 minutes, rather than over a period of hours, as was the case in the early reassembly experiments in which protein had been used in the disaggregated form (6).

The notion that discs are involved in the natural biological process of initation was strengthened by companion experiments (20) in which assembly was carried out with RNAs from different sources. These showed a preference, by several orders of magnitude, of discs for the viral RNA over foreign RNAs or synthetic polynucleotides of simple sequence. It is thus the disc state of the protein that is needed to achieve specificity in the interaction with the RNA. In the experiments cited earlier, in which virus-like rods were made containing TMV A-protein and foreign RNA (8), reactions were carried out at an acid pH, and under these artificial conditions the protein alone would tend to form helical rods and so could entrap any RNA present.

Besides this effect of discs on the rate of initiation, which had been predicted, we also found to our surprise that the discs appeared to enhance the rate of elongation, and we concluded that they must be therefore actively involved in growth. This result has been questioned by some other workers in the field and is still the subject of argument (21, 22), but recent discoveries on the configuration of RNA during incorporation into a growing particle, discussed below, have made the involvement of discs in the elongation, as well as in nucleation, much more intelligible.

The disc form of the protein therefore provided the elements which were missing from the simple reconstitution experiments using disaggregated pro-



Fig, 6. Postulated secondary structure ofthr RNA in the nucleation region (24). This gives a weakly bonded double-helical stem and a look at the top probably the actual origin of assembly. The sequence at and near the top contains a repeating motif of three bases having G in the middle position and A, or U in the outer positions.

tein, namely speed and specificity. We now knew what the disc did, the next question was how did it do it?

The interaction of the protein disc with the initiation sequence on the RNA

Specificity in initiation ensures that only the viral RNA is picked out for coating by the viral protein. This must be brought about by the presence of a unique sequence on the viral RNA for interaction with the protein disc. Zimmern and Butler isolated the nucleation region containing this site by supplying limited quantities of disc protein, sufficient to allow nucleation to proceed, but not subsequent growth, then digesting away the uncoated RNA with nuclease (23, 24). With the varying protein: RNA ratios and different digestion conditions, they found they could isolate a series of RNA fragments, all of which contained a unique common core sequence with variable extents of elongation at either end. These fragments could be rebound to the coat protein when it was in the form of discs. Among this population of fragments was a fragment only about 60 nucleotides long - just over the length necessary to bind round a single disc - and it appeared to represent the minimum protected core. Because of the strong rebinding of this fragment back to the disc, it seemed likely that it constituted the "origin of assembly", where the normal nucleation reaction began.

However, the work on the RNA produced, in turn, another puzzle: the obvious expectation that the nucleation region would be near one end of the RNA turned out to be wrong. The nucleation occurs about one sixth of the way along the RNA from the 3′ end (25), so that over 5000 nucleotides have to be

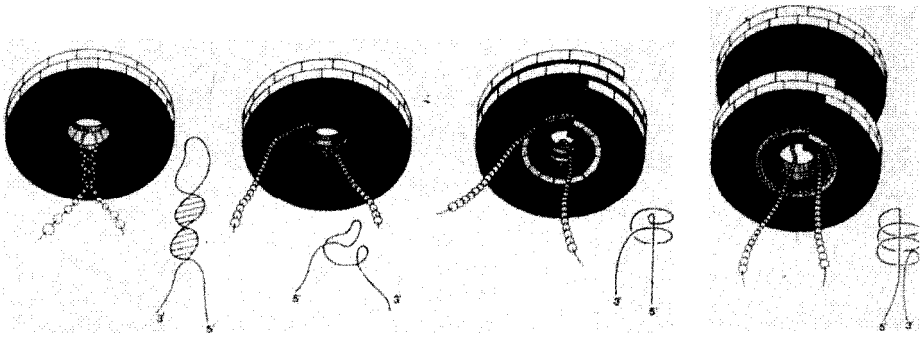


Fig. 7. Nucleation of virus assembly occurs by the insertion of a hairpin of RNA (Fig. 6) into the central hole of the protein disk and betwecn the two layers of subunits. The loop at the top of the hairpin binds to form part of the first turn, opening up the base-paired stem as it does so, and causes the disk to dislocate into a short helix. This presumably "closes the jaws", entrapping the RNA between the turns of protein subunits, and gives a start to the nucleoprotein helix (which can then elongate rapidly to some minimum stable size).

coated in the major direction of elongation (3′-5′) and 1000 have to be coated in the opposite direction. Yet growing nucleoprotein rods observed in the electron microscope (20) were always found to have all the uncoated RNA only at one end: why were rods never seen with a tail at each end? The resolution of this conundrum came from considering the structure of the protein disc, to which I now turn.

Although the structure of the disc was solved in detail only in 1977, an earlier stage in the X-ray analysis gave the clue as to how it might interact with the RNA. At 5 Å resolution (26) the course of the polypeptide chain could be traced and the basic design of the disc established (cf Fig. 4). The subunits of the upper ring of the disc lie in a plane perpendicular to the disc axis while those of the lower ring are tilted downward towards the centre, so that the two rings touch only towards the outside of the disc. In the neighbourhood of the central hole they are thus far apart, like an open pair of jaws which could, as it were, "bite" a stretch of RNA entering through the central hole. Moreover, entry through the centre would be facilitated because the inner region of the protein, from around the RNA binding site inward, was found to be disordered and not packed into a regular structure.

It therefore looked very much as though the disc were designed to permit the RNA to enter through the central hole, effectively enlarged by the flexibility of the inner loop of protein, and intercalate between its two layers. The RNA which would enter thus would of course be the nucleation sequence which lies rather far from an end of the RNA molecule. This could, however, be achieved if the RNA doubled back on itself at a point near the origin of assembly and so entered as a hairpin loop. Indeed, the smallest RNA fragment that is protected during nucleation has a base sequence which can fold into a weakly paired double-helical stem with a loop at the top, that is a hairpin (Fig. 6). This was proposed by Zimmern (24). The loop and top of the stem have an unusual

sequence, containing a repeating motif of three nucleotides, with guanine G in one specific position, and usually A or some times U in the other two. Since there are three nucleotide binding sites per protein subunit, such a triplet repeat pattern will place a specific base in a particular site on the protein molecule and could well lead to the recognition of the exposed RNA loop by the disc during the nucleation process.

Nucleation and growth

The hypothesis for nucleation (27) then is that the special RNA hairpin would insert through the central hole of the disc into the jaws formed by the two layers of protein subunits (Fig. 7). The dimensions are quite suitable for this to occur and the open loop could then bind to the RNA binding sites on the protein. More of the rather unstable double helical stem would melt out and be opened as more of the RNA was bound within the jaws of the nucleating disc. Some, as yet unknown, feature of this interaction would cause the disc to dislocate into a short helical segment, entrapping the RNA and, after the rapid addition of a few more discs (23), would provide the first stable nucleoprotein particle.

The subsequent events after nucleation can be called growth and as stated above there is a controversy about the particular way in which this proceeds. Our view is that elongation in the major direction of growth very likely takes place through the addition of further discs, as indeed our first reconstitution experiments drove us to conclude. The special configuration generated during the insertion of the loop into the centre of the disc must be perpetuated as the rod grows, by pulling further RNA up through the central hole. Thus, elongation could occur by a substantially similar mechanism to nucleation, only now, rather than requiring the specific nucleation loop of the RNA, it occurs by means of a "travelling loop" which can be inserted into the centre of the next incoming disc. This mechanism therefore overcomes the main difficulty in envisaging how a whole disc of protein subunits could interact with the RNA in the growing helix. There is now more evidence for growth by incorporation of blocks of subunits of roughly disc size (22), but the subject is still controversial and I will therefore not proceed further with it.

On the other hand, there is now clear experimental confirmation of our hypothesis for the mechanism of nucleation. This predicts (1) that two tails of the RNA will be left at one end of the growing nucleoprotein rod formed, and (2) that one of these tails would project directly from one end but the other would be doubled back all the way from the active growing point at the far end of the rod down the central hole of the growing rod. Both of these predictions have now been confirmed. Hirth's group in Strasbourg has obtained electron micrographs of growing rods in which the RNA is spread by partial denaturation, and many particles show two tails protruding from the same end (28). In Cambridge my colleagues have used high resolution electron microscopy, in which the two ends of the rods can be identified by their shapes to show that it is indeed the longer tail that is doubled back through the growing rod (29). Other experiments show that the RNA configuration has a substantial effect on the rate of assembly (29).

Design and construction: physical and biological requirements

We have seen that the formation of the protein disk is the key to the mechanism of the assembly of TMV. The protein subunit is designed not to form an endless helix, but a closed two-layer variant of it, the disc, which is stable and which can be readily converted to the lockwasher or helix-going form. The disc therefore represents an intermediate sub-assembly by means of which the entropically difficult problem of nucleating helical growth is overcome. At the same time the nucleation by the disc sub-assembly furnishes a mechanism for recognition of the homologous viral RNA (and rejection of foreign RNAs) by providing a long stretch of nucleotide binding sites for interaction with the special sequence of bases on the RNA. The disc is thus an obligatory intermediate in the assembly of the virus, which simultaneously fulfils the physical requirement for nucleating the growth of the helical particle and the biological requirement for specific recognition of the viral RNA. TMV is self-assembling, self-nucleating and self-checking.

There are a number of morals to be derived from the story of TMV assembly (1). The first is that one must distinguish between the design of a structure and the construction process used to achieve it. That is, while TMV looks like a helical crystal and its design lends itself to a process of simple addition of subunits, its construction actually follows a more complex path that is highly controlled. It illustrates the point that function is inextricably linked with structure and how much can be done by one single protein. A most intricate structural mechanism has been evolved to give the assembly an efficiency and purposefulness whose basis we now understand. The general moral of all this is that not merely does nature once again confound our obvious preconceptions, but it has left enough clues for us to be able to puzzle out finally what is happening. As Einstein once put it, "Raffmiert ist der Herr Gott, aber bösartig ist er nicht: The Lord is subtle, but he is not malicious".

CRYSTALLOGRAPHIC OR FOURIER ELECTRON MICROSCOPY

In 1955, Finch and I in London, and Caspar, then in Cambridge, took up the X-ray analysis of crystals of spherical viruses. These had first been investigated by Bernal and his colleagues just before and after the war, using "powder" and "still" photography. Finch and I worked on Turnip Yellow Mosaic virus and its associated empty shell, and Caspar on Tomato Bushy Stunt virus. Crick and Watson had predicted that spherical viruses ought to have one of the forms of cubic symmetry, and we showed that both viruses had icosahedral symmetry. Later, when Finch and I showed that poliovirus also had the same symmetry, we realised that there was some underlying principle at work, and this eventually led Caspar and me to formulate our theory of virus shell structure (30).

When my research group moved to Cambridge in 1962, we turned to electron microscopy for the speed with which it enables one to tackle new subjects, and also because it produces a direct image, or so we thought. Armed with a theory of virus design and some X-ray data, we had some notion of how spherical shells of viruses might be constructed and thought we would be able

to see the fine detail in electron micrographs. Thus, we knew what we were looking for, but we soon found that we did not understand what we were looking at: the micrographs did not present simple direct images of the specimens. We soon discovered the limitations of electron microscopy. First, there were preparation artefacts and also radiation damage during observation. Secondly, artificial means of contrast enhancement had to be used as the majority of atoms in biological specimens have an atomic number too low to give sufficient contrast on their own. Thirdly, the image formed depends on the operating conditions of the microscope and on the focussing conditions and aberrations present. Above all, because of the large depth of focus of the conventional microscope, all features along the direction of view are superimposed in the image. Finally, in the case of strongly scattering or thick specimens, there is multiple scattering within the specimen, which can destroy even this relation between object and image.

For these reasons, the detail one sees in a raw image is often unreliable and not easily interpretable without methods which correct for the operating conditions of the microscope and which can separate contributions to the image from different levels of the specimen. It is also important to be able to assess the degree of specimen preservation in each particular case. These procedures for image processing of electron micrographs were developed by myself and my colleagues over a period of about 10 years. Their aim is to extract from the information recorded in electron micrographs the maximum amount of reliable information about the 2- or 3-dimensional structures which are being examined. Some applications of these methods to various problems studied in the MRC laboratory over the first 15 years are given in Table 1. Electron microscopy combined with image reconstruction, supplemented wherever possible

Table 1. Some applications of electron microscope image reconstruction in the MRC Laboratory of Molecular Biology, Cambridge, 1964–1979.

Viruses	Organelles	Enzymes, etc.
Helical TMV, TMV protein disc, Paramyxoviruses	Microtubules from flagellar doublets and brain; tubulin sheets	Haemocyanin
Icosahedral Polyoma, wart, TBSV, TYMV, R17, Nudaurelia, CPMV	Muscle filaments: actin; actin + tropomyosin; actin + myosin + tropomyosin (inhibited and relaxed)	Glutamate dehydrogenase
Adenovirus hexon Aberrant hex. & pent tubes of polyoma	Bacterial flagella; Bacterial cell walls	Catalase; crystals and tubes
Phage T2 and T4 Head and its tubular variants (polyheads)	Ribosome crystals	Sickle cell haemoglobin fibres
Tail: sheath + core Baseplate	Chromatin: crystals of nucleosome cores; tubes of histone octamers	Purple membrane (Bacteriohodopsin)
	Gap junctions	Cytochrome oxidase

by X-ray studies on wet, intact material, has provided what are now generally accepted models of the structural organisation of a large number of biological systems such as those listed in the table. In this lecture I will describe a limited number of examples which serve to demonstrate the power of various techniques and the nature of the results they can give. Fuller accounts of the methods and the theory are given elsewhere (2, 3), but I would like to emphasize here that these methods arose out of practical concerns and grew in the course of tackling concrete problems; nervertheless they have proved to be of wide application.

Two dimensional reconstruction: digital computer processing

We began our studies on viruses, both spherical and helical, using the method of negative staining which had been recently introduced by Huxley, and by Brenner and Horne (31). In this method the specimen is embedded in a thin amorphous layer of a heavy metal salt which simultaneously preserves and maps out the shape of the regions from which it is excluded. Much fine detail was to be seen, but one could not easily make sense of it in most cases. People simply thought that the specimens were being disordered, because it was assumed that the negative stain gave, as it were, a footprint of the particle. We gradually came to realise that the confusion arose, not so much because of the disorder that the stain produced, but because there was a superposition of detail from the front and back of the particle; i. e., the stain was enveloping the whole particle, so forming a cast rather than a footprint. This interpretation was proved in two different ways which proceeded in parallel. First, in the case of the spherical viruses, one could build a model and compute or otherwise display it in projection and we found that this could account for many if not all of the previously uninterpretable images (32). The uniqueness of the model could be proved by tilting experiments in which the specimens on the grid and the model were tilted in the same manner through large angles (cf. Fig. 10, ref. 73). The second approach was applied to helical structures, which are translationally periodic and therefore lend themselves to a direct image analysis, which I shall now illustrate.

Figure 8a shows an electron micrograph of a negatively stained specimen of a "polyhead", which is a variant of the head of T4 bacteriophage, consisting mainly of the major head protein. The particle has been flattened and so its original tubular form lost. The image clearly shows some structural periodicities, but these are difficult to discern and such interpretations used to be left to subjective judgement. I realised that the optical (Fraunhofer) diffraction pattern produced from such an image would allow an objective analysis of all the periodicities present to be made (33). This is shown in Figure 8b. Here clear diffraction maxima can be seen: these fall into two sets which can be accounted for as arising respectively from the near and far sides of the specimen. In this way it was established that the negative stain was producing a complete cast of the particle rather than a one sided footprint of it (33). Since this is a helically periodic structure, the diffraction maxima tend to lie on a lattice and so they pick out genuine repeating features within the structure. In

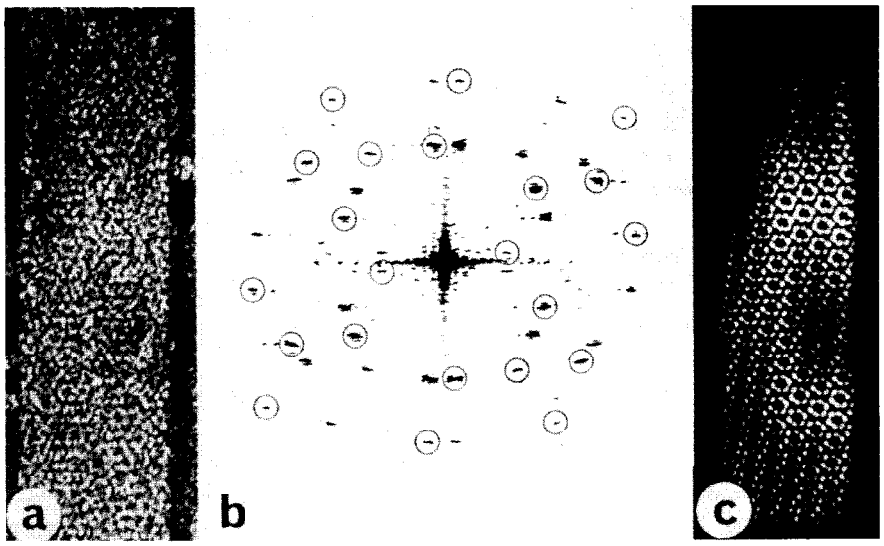


Fig. 8. Optical diffraction and image filtering of thr tubular structures known as "polyheads", consisting: of the major head protein of T4 bactriophagc (35). (a) Electron micrograph of negativcly stained flattened particle x 200,000.

(h) Optical diffraction pattern of (a), with circles drawn around one set of diffraction peaks corresponding to one layer of the structure.

(c) Filtered image of one layer in (a) using the diffaction mask shown in (b). The aperatures in thr mask are chosen so that the averaging here extcnds locally only over a few unit cells. Individual molecules arranged in hcxamers can be seen.

this case the regular diffraction maxima extended to a spacing of about 20 Å which demonstrated that the long range order in the specimen was preserved to this resolution, which is indeed sufficient to resolve individual protein molecules.

The confusion in the image is largely due to the superposition of the near and far sides of the particle, and any one such side can be filtered out in an optical system by a suitably positioned mask which transmits only the desired diffracted rays (34). The filtered image, Figure 8c, is immediately interpretable in terms of a particular arrangement of protein molecules (35).

The clarity of the processed image derives also from the fact that the background noise in the diffraction pattern has been filtered out. This noise arises because of the individual variations between molecules in the specimen, i.e. the disorder, and these contribute randomly in all parts of the diffraction pattern. Indeed, what has been done is that the signal to noise ratio in the image has been enhanced by averaging over the copies of the molecules present in the arrangement. This idea of averaging over many copies of a repeated motif is central to the most powerful techniques developed so far for producing reliable images of biological specimens, and the three dimensional procedures which I will describe later can also use this technique.

The essence of image processing of this type is that it is a two-step procedure after the first image has been obtained. First the Fourier transform of the raw

image is produced. Fourier coefficients are then manipulated or otherwise corrected and then transformed back again to reproduce the reconstructed image. These operations can be carried out most easily on a digital computer, and digital imaging processing as first introduced by DeRosier and myself (36) allows a much greater flexibility than our original optical method and makes three dimensional procedures possible.

Three-dimensional image reconstruction

The first example I have given (Fig. 8) is of a relatively simple case where the problem is essentially that of separating contributions from two overlapping crystalline layers and we have seen how the method of Fourier analysis resolves the superposition in real space into separated sets of contributions in Fourier space. It was, however, already clear from the simple analysis of spherical viruses that in order to get a unique or reliable picture of a three dimensional structure one must be able to view the specimen from very many different directions (32). These different views were often provided by specimens lying in different orientations but they can also be realised by tilting the specimen in the microscope, as mentioned above. Originally, as described above, the different views were interpreted by the building of models, but eventually I saw that a set of transmission images taken in different views could be combined objectively to give a reconstruction of a three-dimensional object.

This happened when DeRosier and I were studying the tail of bacteriophage T4 and our analysis showed that there were contributions to the image from the internal structure as well as from the front and back surfaces (36). To work in three dimensions a generalised form of the two-dimensional filtering process had to be found, and - by making a connection with X-ray analysis - I realised that what is required is a three-dimensional Fourier synthesis. In the analysis of the X-ray diffraction patterns of TMV, I had used the idea that a helical structure could be built up mathematically out of a set of cylindrical harmonic functions; there is a relation between the number of functions that could be obtained and the number of different views available. Each new view would give additional harmonics of higher spatial frequency, and so, if one had enough views, one could build up the complete structure. Later we came to see (36) that this synthesis was only a special case of a general theorem known to crystallographers as the projection theorem.

The general method of reconstruction which we developed (Fig. 9) is based on the projection theorem, which states that the two-dimensional Fourier transform of a plane projection of a three-dimensional density distribution is identical to the corresponding central section of the three-dimensional transform normal to the direction of view. The three-dimensional transform can therefore be built up section by section using transforms of different views of the object, and the three-dimensional reconstruction then produced by Fourier inversion. The important feature of the method is that it tells one how many different views are needed for a required resolution and how these are to be recombined into a three-dimensional map of the object (36, 37). The process is both quantitative and free from arbitrary assumptions. The approach is similar

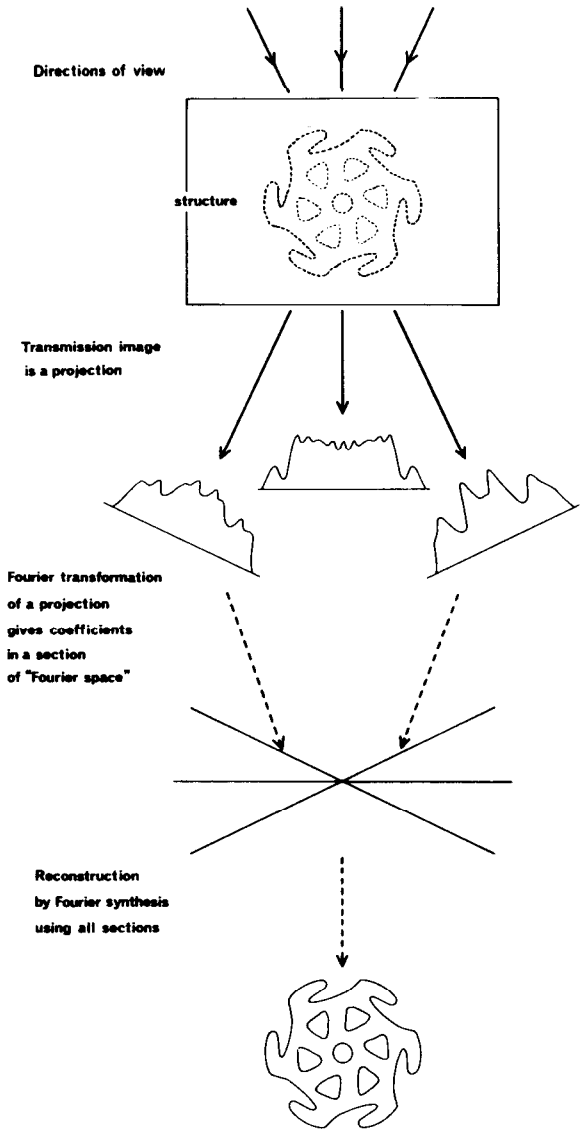


Fig. 9. Scheme for the general process of 3-D reconstruction of an object from a set of 2-D projections (36).

to conventional X-ray crystallography, except that the phases of the X-ray diffraction pattern cannot be measured directly, whereas here they can be computed from a digitised image. Were it not for radiation damage, the different views could be collected from a single particle by using a tilting stage in the microscope, but more realistically one must use several particles in different but identifiable orientations. In general, it is desirable to combine data from different particles so that imperfections can be averaged out.

The Fourier method is only one way out of several for solving the sets of mathematical equations which relate the unknown three dimensional density

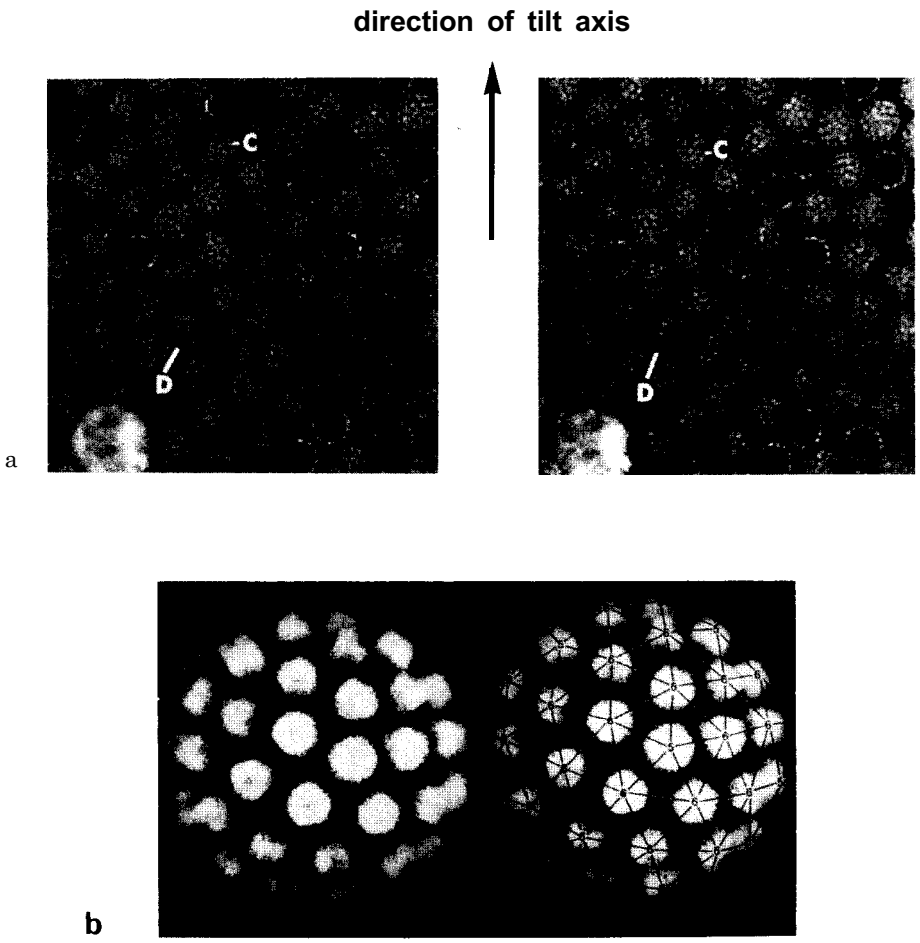


Fig. 10. (a) Electron micrographs of the same field of negatively-stained close-packed particles of human wart virus (HWV) before (i) and after (ii) tilting the specimen grid through an angle close to 18° *(73).* x 100,000.

(b) A three-dimensional reconstructed image of human wart virus (38, 39). Alongside is shown the underlying icosahedral surface lattice (30) with the 5-fold and 6-fold vertices marked.

distribution with known projections in different directions (37), but in fact no other reliable method has been shown to be superior and it is used in the CAT scanner. Moreover, the Fourier method has the advantage that because it is carried out in steps, i.e. formation of the two-dimensional transforms, and then recombination in three dimensions, it is possible as described above, to assess, select, and correct the data going into the final reconstruction.

Many applications have been made. The first application was in fact to the phage tail of T4, the problem in which it had arisen. Particles with helical symmetry are the most straightforward to reconstruct, because a reconstruction can be made from a single view of the whole particle, to a limited resolution, set by the helix symmetry. In physical terms, this is because a single image of a helical particle presents many different views of the repeating

subunit, and it was this simplification that led us to use the phage tail as a first specimen for 3-D image reconstruction. Generally, more than one view is necessary, but any symmetry present will reduce the number required. Typically for small icosahedral viruses, three or four views are sufficient, but many more specimens must be investigated before the appropriate number can be found and averaging carried out (38). An example, from Crowther and Amos (39), is given in Fig. 10.

Phase contrast microscopy

Electron microscopy, combined with some method of image analysis, when applied to negatively stained specimens, has proved ideal for determining the arrangement and shape of small protein subunits within natural or artificial arrays, including two dimensional crystals and macromolecular assemblies such as viruses and microtubules (2). The structural information obtainable has proved to be highly reliable with respect to detail down to about the 20 Å or 15 Å level. It became clear, however, that the degree of detail revealed was limited by the granularity of the negative stain and the fidelity with which it follows the surface of the specimen (40). To obtain much higher resolution information, better than about 10 Å, one should dispense with the stain and view the protein itself. At high resolution, there is a second problem: irradiation damage. This can be reduced by cutting down the illuminating beam, but the statistical noise is then increased, and the raw image becomes less and less reliable. However, this difficulty can be overcome satisfactorily by imaging ordered arrays of molecules, so that the information from the different molecules can be averaged, as described above, to give a statistically significant picture. The first problem of replacing the negative stain, yet avoiding dehydration, can be solved in two ways. One, now being intensively studied, is to use frozen hydrated specimens (41). The second, tried method is that of Unwin and Henderson, who, in their radical approach to determining the structure of unstained biological specimens by electron microscopy (42, 43) used a dried-down solution of glucose to preserve the material.

The question then arose as to how this unstained specimen, effectively transparent to electrons, is to be visualized. In the light microscopy of transparent specimens the well-known Zernike phase contrast method is used. Here the phase of the scattered beams relative to the unscattered beam are shifted by means of a phase plate and then the scattered and unscattered beams are allowed to interfere in the image plane to produce an image. A successful electrostatic phase contrast device for electron microscopy, quite analogous to the phase plate used in light microscopy, was constructed by Unwin (44), but it is not easy to make or use. A practical way of producing phase contrast in the electron microscope is simply to record the image, with the objective lens undcrfocussed, and this was the method used by Unwin and Henderson.

The defocussing phase contrast method arose out of an academic study by Erickson and myself of image formation in the electron microscope (45). This was undertaken because of a controversy that had developed concerning the nature of the raw image itself. When three-dimensional image reconstruction

was introduced and applied to biological particles embedded in negative stain, objections were raised by various workers in the field of materials science, accustomed to dynamical effects in strongly scattering materials, to the premise that the image essentially represented the simple projection of the distribution of stain. It was asked whether multiple or dynamical scattering might not vitiate this assumption. To investigate this question, Erickson and I undertook an experimental study of negatively stained thin crystals of catalase as a function of the depth of focussing (45). We found that a linear or first order theory of image formation would explain almost entirely the changes in the Fourier transform of the image. We concluded that the direct image, using a suitable value of underfocus dependent on the frequency range of interest, is a valid picture of the projection of the object density. When greater values of underfocus were used to enhance the contrast, the image could be corrected to give a valid picture.

This study, although confined to the medium resolution range, included a practical demonstration that *a-posteriori* digital image processing could be used to measure and compensate for the effects of defocussing, and we suggested that this approach could be directly extended to high resolution to compensate for the effects of spherical aberration as well as defocussing. It also provided a convenient way of producing phase contrast in the electron microscope in the case of unstained specimens. The image is recorded with the objective lens underfocussed, so changing the phases of the scattered beams relative to the unscattered (or zero order) beam. Defocussing does not however act as a perfect phase plate analogous to that of Zernike, since the phases are not all changed by the same amount, and successive bands of spatial frequencies contribute to the image with alternately positive and negative contrast. In order to produce a "true" image, the electron image must be processed to correct for the phase contrast transfer of the microscope so that all spatial frequencies contribution with the same sign of contrast.

To produce their spectacular three-dimensional reconstructed image of the purple membrane of Halobacterium to a resolution of about 7 Å (44), Henderson and Unwin took a series of very low-dose images of different pieces of membrane tilted at different angles. The final map represented an average over some 100,000 molecules. The small amount of contrast present in the individual micrographs was produced by underfocussing which was then compensated for in the computer reconstruction by the method described above. For the first time the internal structure of a protein molecule was "seen" by electron microscopy.

THE STRUCTURE OF CHROMATIN

The work on viruses has given results not only of intrinsic interest, but as I indicated above, the difficulties in tackling large molecular aggregates led to the development of methods and techniques which could be applied to other systems. A recent example of this approach, and one which I think would not have gone so fast without our earlier experience, is that of chromatin. Chroma-

tin is the name given to the chromosomal material when extracted. It consists mainly of DNA, tightly associated with an equal weight of a small set of rather basic proteins called histones. We took up the study of chromatin in Cambridge about ten years ago when the protein chemists had shown that there were only five main types of histones, the apparent proliferation of species being due to post-synthetic modifications, so that the structural problem appeared tractable.

The DNA of the eukaryotic chromosome is probably a single molecule, amounting to several centimeters in length if laid out straight, and it must be highly folded to make the compact structure one can see in a chromosome. At the same time it is organised into separate genetic or functional units, and the manner in which this folding is achieved, genes organised and their expression controlled, is the subject of intense study throughout the world. The aim of our research group has been to try to understand the structural organisation of chromatin at various levels and to see what connections could be made with functional controls.

The large amounts in which histones occur suggested that their role was structural, and it was shown over the years 1972-1975 that the four histones H2A, H2B, H3 and H4 are responsible for the first level of structural organisation in chromatin. They fold successive segments of the DNA about 200 base pairs long into compact bodies of about 100 Å in diameter, called nucleosomes. A string of nucleosomes or repeating units is thus created and when these are closely packed they form a filament about 100 Å in diameter. The role of the fifth histone H1 was at first not clear. It is much more variable in sequence than the other four, being species and tissue specific. In the years 1975-1976 we showed that HI is concerned with the folding of the nucleosomc filament into the next higher level of organisation, and later how it performed this role.

This is not the place to tell in detail how this picture of the basic organisation of chromatin emerged (4), but the idea of a nucleosome arose from the convergence of several different lines of work. The first indications for a regular structure came from X-ray diffraction studies on chromatin which showed that there must be some sort of repeating unit, albeit not well ordered, on the scale of about 100 Å (46, 47). The first biochemical evidence for regularity came from the work of Hewish and Burgoyne (48) who showed that an rndogenous nuclease in rat liver could cut the DNA into multiples of a unit size, which was later shown by Noll, using a different enzyme, micrococcal nuclcasc, to be about 200 base pairs (49). The fact that the nuclcasc cuts the DNA of chromatin at regularly spaced sites, quite unlike its action on free DNA, is attributed to the fact that the DNA is folded in such a way as to make only short stretches of free DNA between these folded units available to the enzyme. The third piece of evidence which led to the idea of a nuclcosome was the observation by Kornberg and Thomas (50) that the two highly conserved histones, H3 and H4, existed in solution as a specific oligomrr, the tetramer $(H3)_2(H4)_4$, which behaved rather like an ordinary multi-subunit globular protein. On the basis of these different lines of evidence, Kornbrrg in 1974 (51) proposed a definite model for the basic unit of chromatin as a bead of about 100 A diameter,

containing a stretch of DNA 200 base pairs long condensed around the protein core made out of 8 histone molecules, namely the $(H3)_2(H4)_2$ tetramer and 2 each of H2A and H2B. The fifth histone, H1, was somehow associated with the outside of each nucleosome. A quite unexpected feature of the model was that it was the DNA which "coated" the histones, rather than the reverse.

However, in 1972, when Kornberg came to Cambridge, all this lay in the future. We began using X-ray diffraction to follow the reconstitution of histones and DNA, because the X-ray pattern given by nuclei, or by chromatin isolated from them, limited as it was, was the only assay then available to follow the ordered packaging of the DNA. These X-ray studies showed that almost 90% reconstitution could be achieved when the DNA was simply mixed with an unfractionated total histone preparation, but all attempts to reconstitute chromatin by mixing DNA with a set of all four purified single species of histone failed, as if the process whereby the histones were being separated was denaturing them. We therefore looked for milder methods of histone extraction and found that the native structure could be reformed readily if the four histones were kept together in two pairs, H3 and H4 together, and H2A and H2B together, but not once they had been taken apart. It was this work which led Kornberg to investigate further the physicochemical properties of the histones and to the discovery (50) of the histone tetramer $(H3)_2(H4)_2$, which in turn led him to the model of the nucleosome as described above.

The structure of the nucleosome

Approaches such as nuclease digestion and X-ray scattering on unoriented specimens of chromatin or nucleosomes in solution could reveal certain features of the nucleosome, but a full description of the structure can only come from crystallographic analysis, which gives complete three-dimensional structural information. In the summer of 1975 my colleagues and I therefore set about trying to prepare nucleosomes in forms suitable for crystallisation. Nucleosomes purified from the products of micrococcal nuclease digestion contain an average of about 200 nucleotide pairs of DNA, but there is a rather wide distribution about the average, and such preparations are not homogeneous enough to crystallize. However, this variability in size can be eliminated by further digestion with micrococcal nuclease. While the action of micrococcal nuclease on chromatin is first to cleave between nucleosomes, it subsequently acts as an exonuclease on the excised nucleosome, shortening the DNA first to about 166 base pairs, where there is a brief pause in the digestion (52), and then about 146 base pairs, where there is a clear plateau in the course of digestion, before more degradation occurs. During this last stage the histone Hl is released (52), leaving as a major metastable intermediate a particle containing 146 base pairs of DNA complexed with a set of 8 histone molecules. This enzymatically reduced form of the nucleosome is called the core particle and its DNA content was found to be constant over many different species. The DNA removed by the prolonged digestion, which had previously joined one nucleosome to the next, is called the linker DNA.

A core particle therefore contains a well-defined length of DNA and is

homogeneous in its protein composition. We naturally tried to crystallize preparations of core particles, but we were not at first successful probably because of small traces of the fifth histone Hl. Eventually my colleague Leonard Lutter found a way to produce exceptionally homogeneous preparations of nucleosome core particles, and these formed good single crystals (53). The conditions for growing the crystals were based on our previous experience in crystallising transfer-RNA, because we reasoned that a good part of the nucleosome core surface would consist of DNA. These experiments perhaps surprised biologists in showing dramatically that almost all the DNA in the nucleus is organised in a highly regular manner.

The derivation of a three-dimensional structure from a crystal of a large molecular complex is, as for the TMV disk, a process that can take many years. We have therefore concentrated on obtaining a picture of the nucleosome core particle at low resolution by a combination of X-ray diffraction and electron microscopy, supplemented where possible by biochemical and physicochemical studies. We first solved the packing in the crystals by analysing electron micrographs of thin crystals and then obtained projections of the electron density along the three principal axes of the crystals, using X-ray diffraction amplitudes and electron microscope phases (53, 54). The nucleosome core particle turned out to be a flat disc-shaped object, about 110 Å by 110 Å by 57 Å, somewhat wedge-shaped, and strongly divided into two layers. We proposed a model in which the DNA was wound into about $1^3/_4$ turns of a shallow superhelix of pitch about 27 Å around the histone octamcr. There are thus about 80 nucleotides in each turn of the superhelix. This model for the organisation of DNA in a nucleosome core also provided an explanation for the results of certain enzyme digestion studies on chromatin (53, 55) thus showing that what we had crystallised was essentially the native structure.

The first crystals we obtained were found to have the histone proteins within them partly proteolysed, but their physicochemical properties remained very similar to those of the intact particle. We have since grown crystals from intact nucleosome cores which diffract to a resolution of about 5 Å and a detailed analysis is in progress (56). Over the years Daniela Rhodes, Ray Brown and Barbara Rushton have grown crystals of core particles prepared from seven different organisms: all give essentially identical X-ray patterns testifying to the universality of nucleosomes. There is a dyad axis of symmetry within the particle, which is not surprising since the 8 histones occur in pairs and DNA is studded with local dyad axes. High angle diffuse X-ray scattering from the crystals shows that the DNA of the core particle is in the B-form.

An electron density map of one of the principal projections of the crystal is shown in Fig. 11a. This map gives the total density in the nucleosome, the density of the DNA not being distinguished from that of the protein. The contributions of protein and DNA can be distinguished by using neutron scattering combined with the method of contrast variation and such a study was therefore begun by John Finch and a group at the Institute Laue Langevin, Grenoble, when sufficiently large crystals were available (57). They obtained maps of the DNA and protein along the three principal projections (see Figs.

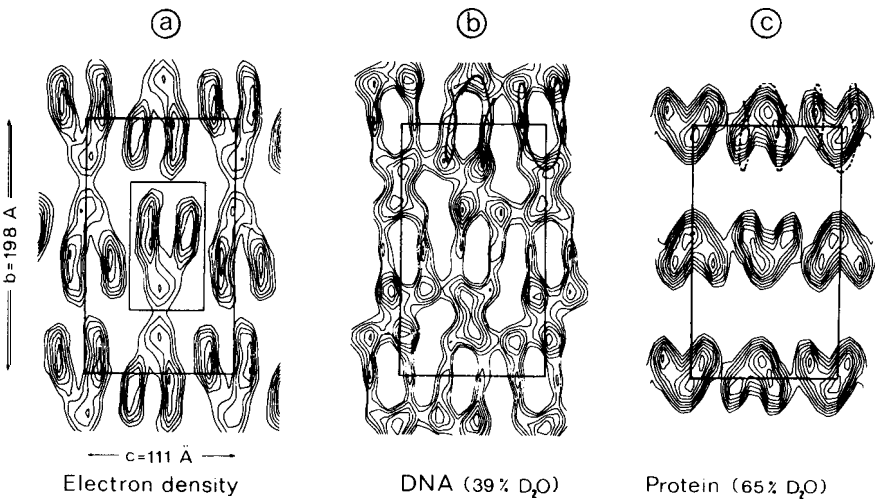


Fig. II. Fourier projection maps of the nucleosome core particle. (a) Map from X-ray data (56); (b) and (c) from neutron scattering data using contrast variation (57): (b) the DNA component with the path of the superhelix drawn superimposed on the density; (c) the protein core component.

11b and c). The map of the DNA is consistent with the projection of about $1^3/_4$ superhelical turns as proposed earlier, and the map of the protein shows that the histone octamer itself is consistent with a wedge shape.

Three dimensional image reconstruction of the histone octamer and the spatial arrangement of the inner histones

An alternative to separating the contributions of the DNA and the protein by neutron diffraction is to study the histone octamer directly. The histone octamer which forms the protein core of the nucleosome can exist in that form free in solution in high salt, which displaces the DNA (58). In the course of attempts to crystallize it, we obtained ordered aggregates - hollow tubular structures - which were investigated by electron microscopy (59). The image reconstruction method described above was used to produce a low resolution three-dimensional map and model of the octamer (fig. 12a). As a check that the removal of DNA had not led to a change in the structure of the histone octamer, projections of this model were calculated and compared with the projections of the protein core of the nucleosome obtained from the neutron scattering study mentioned above. There was a good agreement between the three maps showing that the gross structure was not altered.

To the resolution of the anlysis (20 Å) it was shown that the histone octamer possesses a two-fold axis of symmetry, just as does the nucleosome core particle itself. Like the nucleosome core, the histone octamer is a wedgeshaped particle of bipartite character. Its periphery shows a system of ridges which form a more or less continuous helical ramp of external diameter 70 Å and pitch about 27 Å, exactly suitable for it to act as a spool on which could be wound about $1^3/_4$ turns of superhelix of DNA in the appropriate dimensions (Fig. 12b).

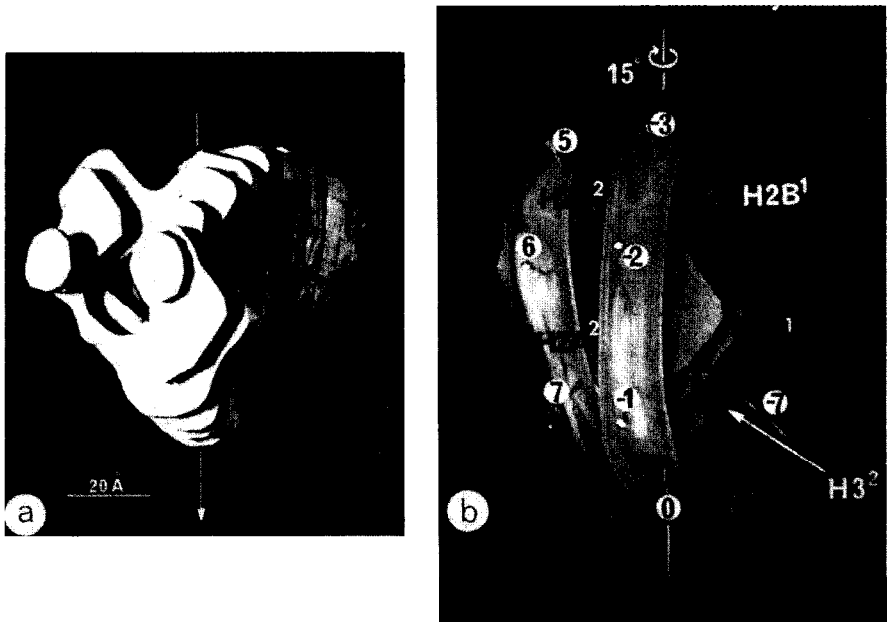


Fig. 12. (a) Model of the histone octamer obtained by three-dimensional image reconstruction from electron micrographs (59). The dyad axis is marked. The ridges on the periphery of the model form a left-handed helical ramp on which 1 3/4 to 2 turns of a superhelix of DNA could be wound.

(b) The histone octamer structure (a) with two turns of a DNA superhelix wound around it. (Note that for clarity, the diameter of the plastic tube has been chosen smaller than the true scale for DNA.) Distances along the DNA are indicated by the numbers -7 to +7, taking the dyad axis as origin, to mark the 14 repeats of the double helix contained in the 146 base pairs of the nucleosome core. The assignment of the individual histones to various locations on the model is described in the text.

The resolution of the octamer map is too low to define individual histone molecules, but we have exploited the relation of the octamer to the superhelix of DNA to interpret them in terms of individual histones (59). This interpretation uses the results of Mirzabekov and his colleagues (60) on the chemical cross-linking of histones to nucleosomal DNA, and also information on histonel histone proximities given by protein crosslinking. This data cannot be interpreted reliably without a three-dimensional model because a knowledge of the points of contact of histones along a strand of the DNA is not sufficient to fix a spatial arrangement of the histones in the nucleosome core. Furthermore, because the two superhelical turns of DNA are close together the pattern of histone/DNA crosslinks need not directly reflect the linear order of histones along the DNA. The three-dimensional density map restricts the number of possiblities and enables choices to be made.

In the spatial arrangement proposed, the helical ramp of density in the octamer map is composed of a particular sequence of the eight histones, in the order H2A-H2B-H4-H3-H3-H4-H2B-H2A, with a dyad in the middle. The $(H3)_2(H4)_2$ tetramer has the shape of a dislocated disc or single turn of a

helicoid, which defines the central turn of a DNA superhelix. The structure for the histone tetramer explains the findings of many workers, expanding on the original observations of Felsenfeld (61), that H3 and H4 alone, in the absence of H2A and H2B, can confer nucleosome-like properties on DNA, in particular supercoiling and resistance to micrococcal nuclease digestion, whereas H2A and H2B alone cannot. It also explains the asymmetric dissociation of the histone octamer when the salt concentration is lowered: the octamer dissociates, through a hexameric intermediate, into a $(H3)_2(H4)_2$ tetramer and two H2A.H2B dimers (58, 62).

The role of H1 and higher order structures

These studies have given a fairly detailed picture of the internal structure of the nucleosome, but until 1975 there was still no clear idea of the relation of one nucleosome to another along the nucleosome chain or basic chromatin filament, nor of the next higher level of organisation. It had been known for some time that the thickness of fibres observed in electron microscopical studies of whole mount chromosome specimens varied from about 100 to 250 Å in diameter, depending on whether chelating agents had been used or not in the preparation. Taking this as a clue, Finch and I carried out some experiments *in vitro* on short lengths of chromatin prepared by brief micrococcal digestion of nuclei (63). In the presence of chelating agents this native chromatin appeared as fairly uniform filaments of 100 Å diameter. When Mg^{++} ions were added, these coiled up into thicker, knobbly fibres about 250-300 Å diameter, which are transversely striated at intervals of about 120-150 Å, corresponding apparently to the turns of an ordered, but not perfectly regular helix or supercoil. Since the term "supercoil" had already been used up in a different context, we called it a solenoid, because the turns were spaced close together. On the basis of these micrographs and companion X-ray studies (64), we suggested that the second level of folding of chromatin was achieved by the winding of the nucleosome filament into a helical libre with about 6 nucleosomes per turn. Moreover, we found that when the same experiments were carried out on H1-depleted chromatin, only irregular clumps were formed, showing that the fifth histone H1 is needed for the formation or stabilisation of the ordered libre structure.

These experiments told us the level at which H1 performs its function of condensing chromatin, but the way in which the H1 molecule mediates the coiling of the 100 Å filament into the 300 Å libre only became clear later by putting together evidence from the biochemistry, from the crystallographic analysis, and from more relined electron microscope observations.

From observations on the course of nuclease digestion, taken in conjunction with the known X-ray structure of the nucleosome core, one can deduce where the Hl might be on the complete nucleosome. I have mentioned that there is an intermediate in the digestion of chromatin by micrococcal nuclease at about 166 base pairs of DNA and it is during this step from 166 to 146 base pairs that Hl is released (52). Since the 146 base pairs of the particle correspond to $1^3/_4$ superhelical turns, we therefore suggested that the 166 base pair particle

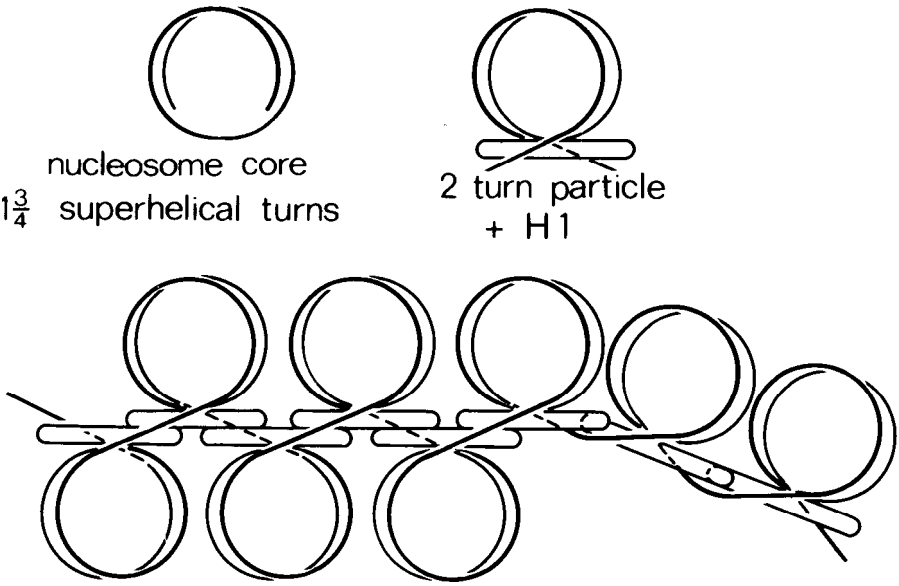


Fig. 13. (Top) If the 146 base pairs of DNA in the nucleosome core correspond to 1 3/4 superhelical turns, then the 166 base particle corresponds to about 2 full superhelical turns. Since the 166 base pair particle is the limit point for the retention of H1 (52), it must be located as shown. (Bottom) Schematic diagram of the nucleosome filament at low ionic strength, showing origin of the zigzag structure (Fig. 14). At the right of the drawing is shown a variant of the zigzag structure which is often observed: this is formed by flipping a nucleosome by 180° about the filament axis.

contains two full turns of DNA (53). This brings the two ends of the DNA on the nucleosome close together so that both can be associated with the same single molecule of H 1 (Fig. 13). A particle consisting of the histone octamer and 166 base pairs has been called the chromatosome (65) and has been suggested by us and others to constitute the basic structural element of chromatin. In this particle, the H 1 would therefore be on the side of the nucleosome in the region of the entry and exit of the DNA superhelix.

This location follows in logic: but was histone H 1 really there? Although H 1 is too small a molecule to be seen directly by electron microscopy, its position in the nucleosome can be inferred from its effect on the appearance of chromatin, in the intermediate range of folding between the 100 A nucleosome filament and the 300 Å solenoidal fibre. These intermediate stages were revealed in the course of a systematic study by Thoma and Koller (66), of the folding of chromatin with increasing ionic strength. By employing monovalent salts rather than divalent ones, they exposed a range of structures showing increasing degrees of compaction as the ionic strength was raised. Thus, from the filament of nucleosomes around 1 mM, the extent of structure increased through a family of intermediate helical structures until, by 60 mM, the compact 300 Å fibre structure was formed, in all respects identical to that originally observed by Finch and myself.

The location of Hl can be deduced by considering the difference between the structures observed in the range of ionic strength l-5 mM in the presence or

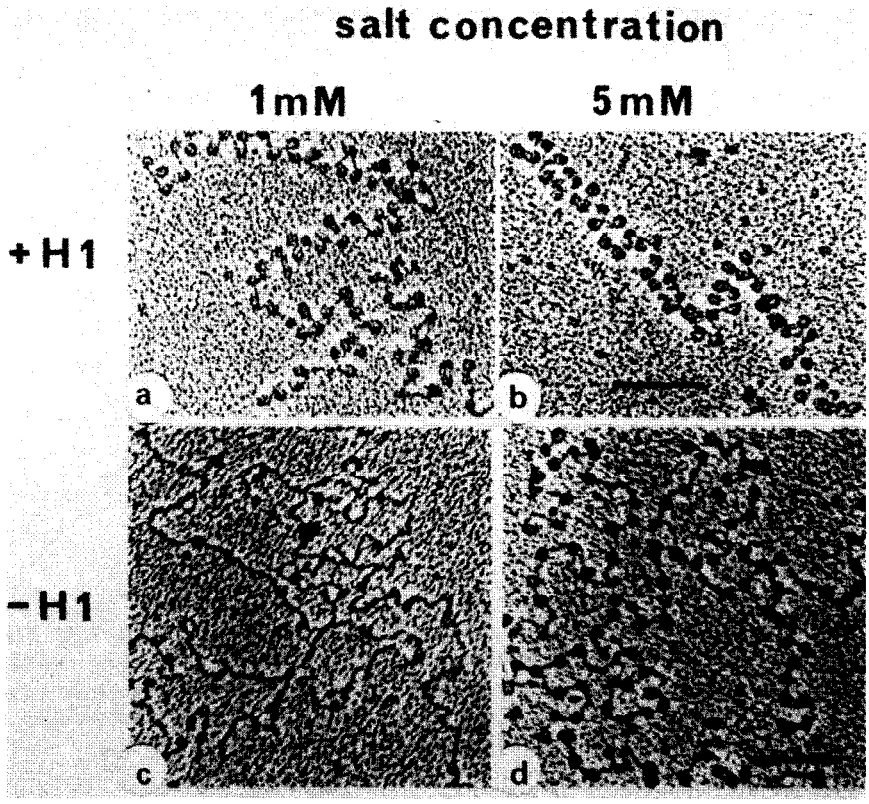
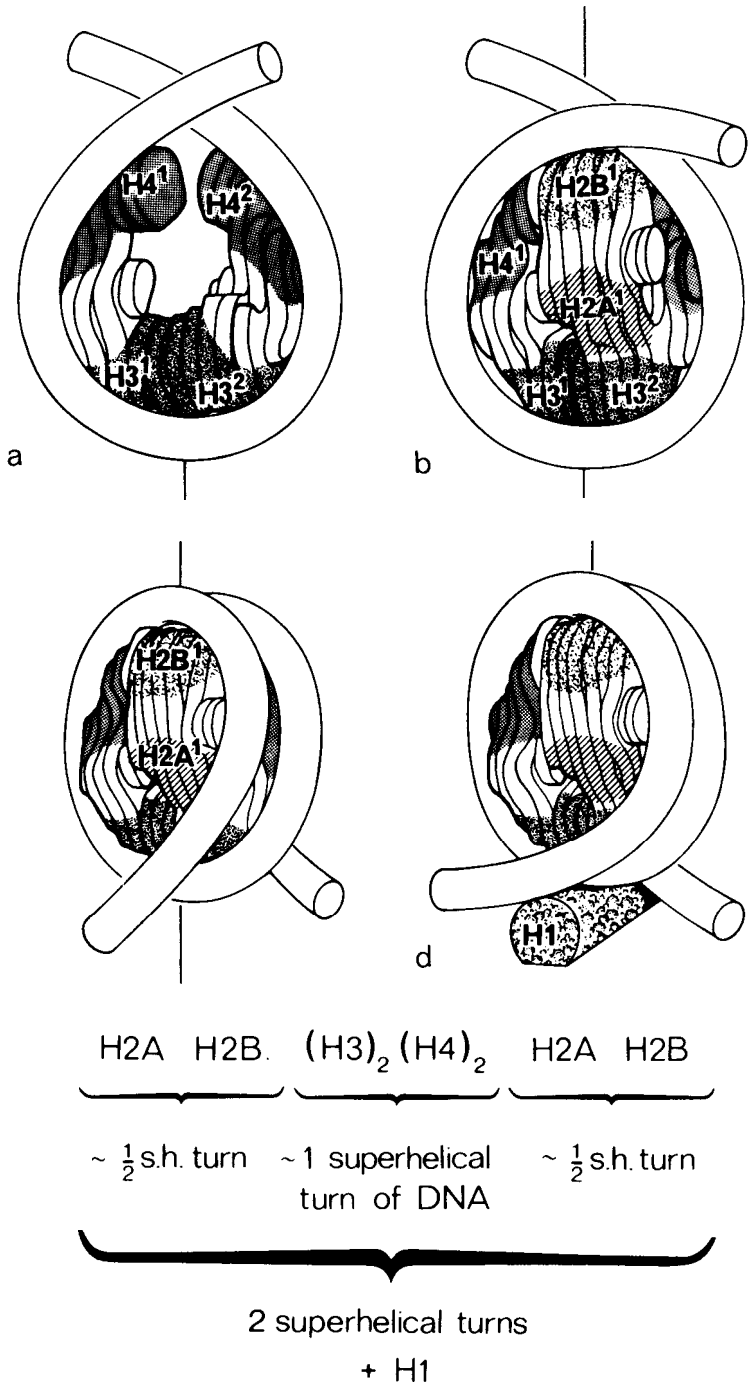


Fig. 14. The appearance of chromatin with and without H1 at low ionic strength (66). When H1 is present the first recognizable ordered structure is (a) a loose zigzag in which the DNA enters and leaves the nucleosomc at sites close together; at a somewhat higher salt concentration (b) the zigzag is tighter. In the absence of H1, there is no order in the sense of a defined filament direction; (c) at the lower salt concentration, nucleosome beads are no longer visible, the structure having opened to produce a fibre of DNA coated with histones; (d) at a higher ionic strength, beads are again visible but the DNA enters and leaves the nucleosome more or less at random. The bar represents 100 nm.

absence of H1 (Fig. 14). In chromatin containing H1, an ordered structure is seen in which the nucleosomes are arranged in a regular zigzag with their flat faces down on the supporting grid. The zigzag form arises because the DNA enters and leaves the nucleosome at sites close together, as one would expect from the combination of X-ray and biochemical evidence mentioned in the last paragraph (Fig. 13). In chromatin depleted of H1, entrance and exit points are more or less on opposite sides and in any case randomly located. Indeed, at very low ionic strength, the nucleosomal structure unravels into a linearised form in which individual beads are no longer seen. When H1 is present this is prevented from happening. We therefore concluded that H1, or strictly part of it, must be located at, and stabilises, the region where DNA enters and leaves the nucleosome, as was predicted.

In the zigzag intermediates the H1 regions on adjacent nucleosomes appear



Fig, 15. "Exploded" views of the nucleosome, showing the roles of the constituent histones. The patches on the histone core indicate locations of individual histone molecules, but the boundaries between them are not known and are thus left unmarked. (a) the $(H3)_2(H4)_2$ tetramer has the shape of a lock washer and can act as a spool for 70-80 b.p. of DNA, forming about one superhelical turn. (b) an H2A.H2B dimer associates with one face of the tetramer. (c) H2A.H2B dimers on opposite faces each bind 30-40 b.p. DNA, or one-half a superhelical turn, to give a complete P-turn particle. (d) histone HI interacts with the unique configuration of DNA at the entry and exit points to seal off the nucleosome.

to be close together or touching. We therefore suggested that, with increasing ionic strength, more of the Hl regions interact with one another, eventually aggregating into a helical polymer along the centre of the solenoid and thus accounting for its geometrical form. Polymers of H1 have indeed been shown to exist by chemical crosslinking experiments at both low and high ionic strength (61), but it remains to be shown that they are located in the centre of the libre. The important point, however, is that it appears to be the aggregation of H1 which accompanies, and indeed may control, the formation of the 300 Å fibre.

The roles of the histones

From the spatial arrangements of molecules proposed for the histone octamer and from the location deduced for histone H1, one can see (59) the roles of the individual histones in folding the DNA on the nucleosome (Fig. 15). The $(H3)_2(H4)_2$ tetramer has the shape of roughly a single turn of a helicoid and this defines the central turn of the DNA superhelix. H2A and H2B add as two heterodimers, H2A.H2B, one on each face of the H3-H4 tetramer, each binding one extra half-turn of the DNA, thereby completing the two-turn superhelix. Finally, H1 then binds to the unique region at the side of the two-turn particle where three segments of DNA come together, stabilizing and "sealing off' the nucleosome, and also mediating the folding to the next level of organisation. Such a sequence of events in time would provide a structural rationale for the temporal order of assembly of histones on to newly replicated DNA (68, 69, 70).

We now have arrived at a moderately detailed model of the nucleosome and a description for the next higher level of folding. There is thus a firm structural and chemical framework in which to consider the dynamic processes which take place in chromatin in the cell, that is, transcription, replication and mitosis.

CONCLUDING REMARKS

I particularly wanted to outline the chromatin work because it may serve as a contemporary paradigm for structural studies which try to connect the cellular and the molecular. One studies a complex system by dissecting it out physically, chemically, or in this case enzymatically, and then tries to obtain a detailed picture of its parts by X-ray analysis and chemical studies, and an overall picture of the intact assembly by electron microscopy. There is, however, a sense in which viruses and chromatin, which I have described in this lecture, are still relatively simple systems. Much more complex systems, ribosomes, the mitotic apparatus, lie before us and future generations will recognise that their study is a formidable task, in some respects only just begun. I am glad to have had a hand in the beginnings of the foundation of structural molecular biology.

Acknowledgements

It will be obvious that I could not have accomplished all that has been summarised here without the help of many highly able and valued colleagues

and collaborators. After Rosalind Franklin's death, I was able to continue and extend the virus work with John Finch and Kenneth Holmes, who were then students, and who became colleagues. Over the years I have had a transatlantic association with Donald Caspar and have benefitted from his advice, criticism and insights. I can mention here only some of the names of my other collaborators in the several branches in which I have been involved: in the study of virus chemistry and assembly, Reuben Leberman, Tony Durham, Jo Butler and David Zimmern; in virus crystallography, William Longley, Peter Gilbert, John Champness, Gerard Bricogne and Anne Bloomer; in electron microscopy and image reconstruction, David DeRosier, Harold Erickson, Tony Crowther, Linda Amos, Jan Mellema, Nigel Unwin and, throughout, John Finch; in the structural studies on transfer RNA, Brian Clark, who provided the biochemical background without which the work could not have begun, Jon Robertus, Jane Ladner and Tony Jack; in chromatin, Roger Kornberg, whose skill and insight transformed a "messy" project into a clear problem, Markus Noll, Len Lutter, and also Daniela Rhodes and Ray Brown, who fruitfully transferred their experience from tRNA to nucleosomes, and finally Tim Richmond and John Finch who are engaged in the higher resolution X-ray studies now in progress.

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