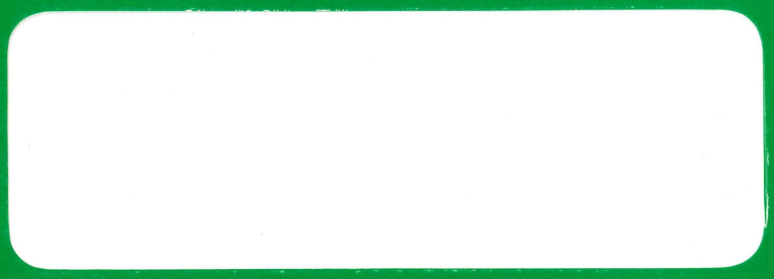

EMBL

RESEARCH REPORTS 1976

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DIVISION OF CELL BIOLOGY

Virus and other membranes

Members: K Simons, H Garoff, A Helenius

Postdoctoral fellows: E Fries, A Ziemiecki

Technical assistants: B Holle*, K Goldmann*, E Kiko, H Virta

We have continued our studies of Semliki Forest virus to probe into the structure, function and synthesis of biological membranes. This animal virus is composed of a spherical nucleocapsid which is assembled in the cytoplasm of the host cell, and a membrane that is acquired when the nucleocapsid buds out from the host cell plasma membrane. Our present view of the structure of the virus membrane is mainly based on biochemical and electronmicroscopical studies (Plate 1), but we hope to extend this schematic structure to atomic resolution, since the virus can form three-dimensional crystals. Semliki Forest virus apparently enters its host cell by fusion of the virus membrane with the host cell plasma membrane. How the fusion between the two membranes is brought about is not yet understood at the molecular level. We know that the spike glycoproteins (composed of three polypeptides E_1, E_2 and E_3) are essential for the fusion. Studies to define the role of the virus membrane polypeptides in the virus-cell fusion process have recently begun.

We have also started work on another membrane protein system, namely penicillinase from *Bacillus licheniformis*. This enzyme is an important cause of drug resistance to penicillin. More important for our purpose is that the membrane-bound form of the penicillinase is a precursor of the secreted water-soluble form of the enzyme. We want to study this protein as a model for protein secretion: how is the enzyme transferred through the bacterial cell membrane? We have chosen a bacterial protein mainly because we want to use genetical methods in addition to biochemistry to dissect the secretory process.

A third aspect of our studies is to develop methods for handling membrane proteins. This is still a major bottleneck in membrane research.

Semliki Forest virus

Structure and synthesis

The subunit structure of the spikes on the virus has been elucidated by the combined use of cleavable protein cross-linking reagents, antibodies against E_1 and E_2, and detergent solubilization. We found that each spike consists of a trimer of E_1, E_2 and E_3. We have also been able to show that the spike proteins are attached to the surface of the nucleocapsid by protein-protein interactions. We do not yet know whether both E_1 and E_2 penetrate the membrane (E_3 is definitely on the outside), nor do we know the orientation of E_1 and E_2: are the N-termini inside or outside? Studies are in progress to answer these questions using protease

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* part of year

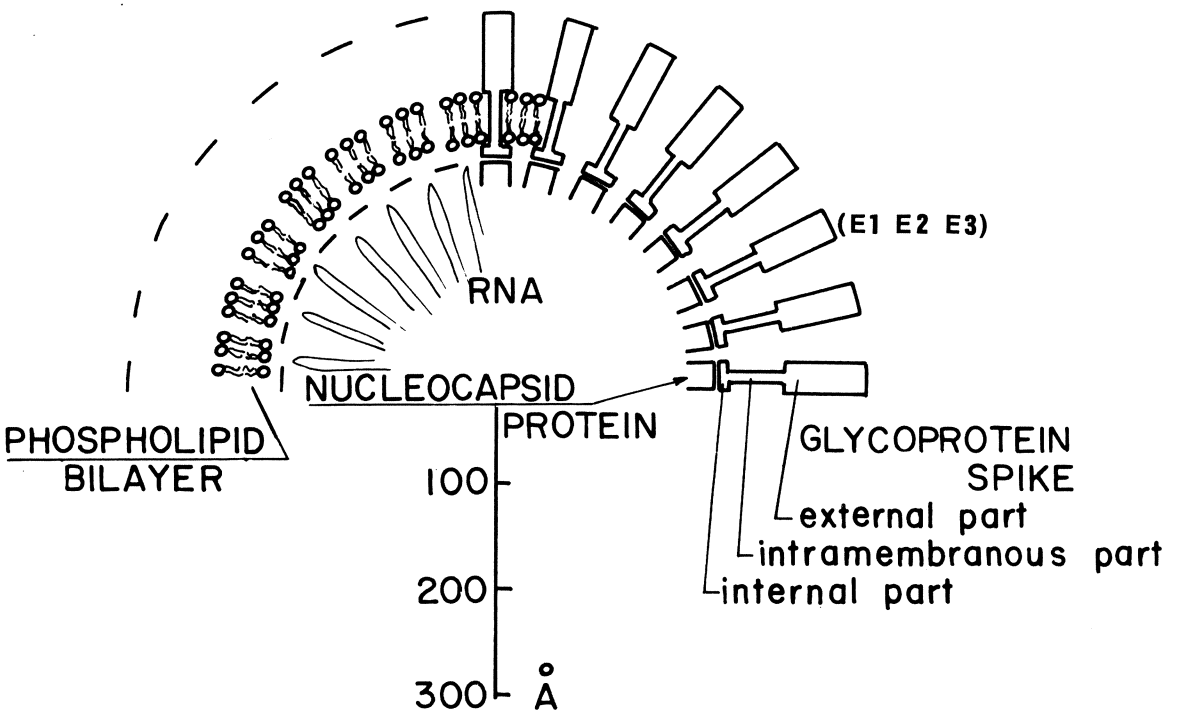

Plate 1

Schematic cross-section of the structure of Semliki Forest Virus

cleavage and micro-amino-acid sequencing. Analyses of electron micrographs of virus pelleted by ultracentrifugation (by D Wiley, Boston and C-H Bonsdorff, Helsinki) have shown that the virus forms three-dimensional crystals with the space group *F*23. The size of the largest crystals found so far is about 20 microns. In collaboration with R Leberman we are attempting to obtain crystals of the virus suitable for x-ray crystallography.

All four polypeptides of the virus, the nucleocapsid protein (NC) and the spike polypeptides E_1, E_2 and E_3, are synthesized as a single polypeptide chain of molecular weight 130,000. The order of the polypeptides in this precursor is, from the N-terminus: NC - (E_3E_2) - E_1. The NC polypeptide is cleaved off the nascent chain and remains in the cytoplasm whereas the E polypeptides are inserted into the endoplasmic reticulum. In collaboration with B Dobberstein, we will attempt to find out whether the cleavage of the NC protein reveals a signal sequence on the exposed N-terminus of the $E_3E_2E_1$ part of the precursor protein for binding to the endoplasmic reticulum, such as has been found for secretory proteins. These studies will be based on *in vitro* translation and on micro-amino acid sequencing.

Solubilization and reconstitution

In addition to the detergents previously studied (Triton X-100, deoxycholate and SDS), we have now investigated a new non-ionic detergent, octylglucoside, which has a critical micellar concentration two orders of magnitude higher than that of Triton X-100. The solubilization sequence of the virus membrane is similar to that found for the other detergents. One interesting finding is that, by manipulating the pH and the ionic strength, the viral lipids can be solubilized leaving most of the spikes attached to the viral nucleocapsids. Octylglucoside has furthermore proved to be nearly ideal for reconstitution studies. The solubilized lipid-free spike protein can easily be reconstituted together with added lipids to form vesicles with the spikes oriented outwards (Plate 2A).

Penicillinase from *Bacillus licheniformis*

Structure and synthesis

(This work is being done in collaboration with M Sarvas, Helsinki). We have purified the membrane form and the secreted water-soluble so-called exo form of the penicillinase. The exo form is well known from earlier studies. It has a molecular weight of 2.9×10^4 and its amino acid sequence has been determined. Our studies show that the membrane-bound form of the enzyme appears to carry an additional tail of about 60 amino acids which is hydrophobic. This tail is presumably cleaved off by a protease when the exo form is released from the membrane during secretion. The purified lipid-free hydrophobic form can be reassociated with lipids to form asymmetric vesicles (Plate 2B). Octylglucoside was also used in these reconstitution studies.

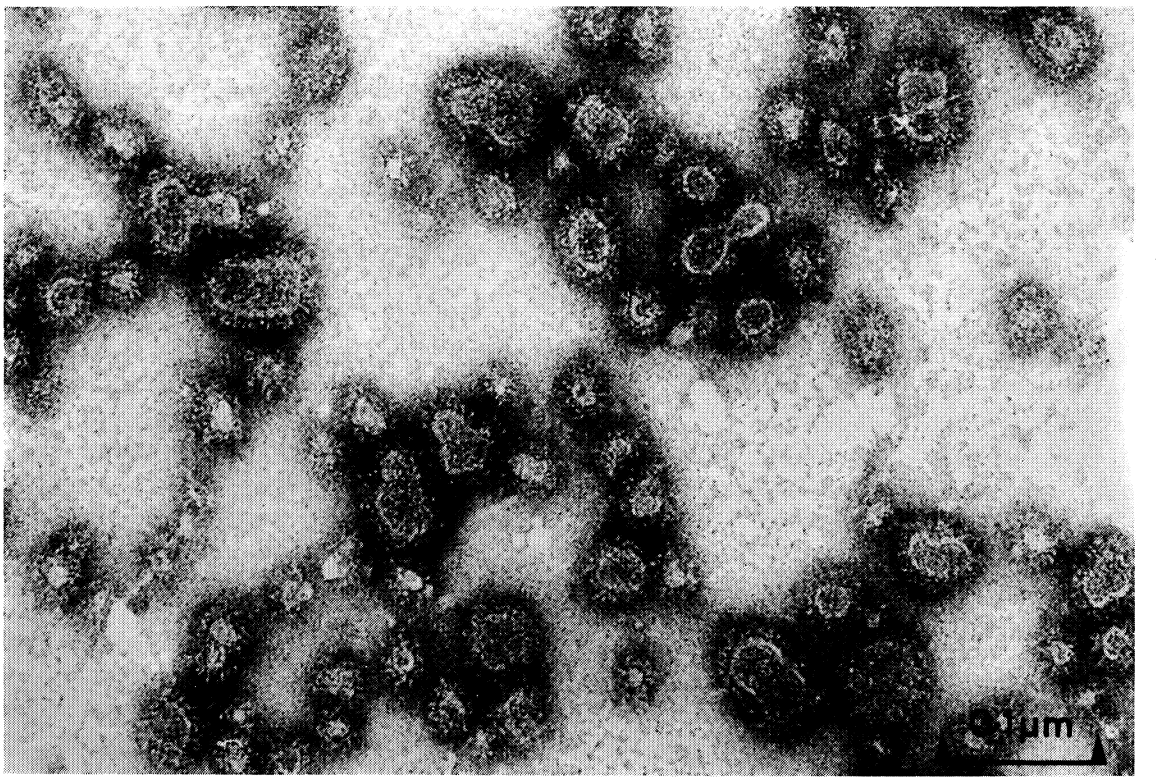

Plate 2A

Reconstituted vesicles of egg phosphatidylcholine and the Semlike Forest virus membrane glycoproteins E_1, E_2 and E_3.

The membrane proteins were delipidated and then added to the lipids in octylglucoside. The detergent was removed by dialysis.

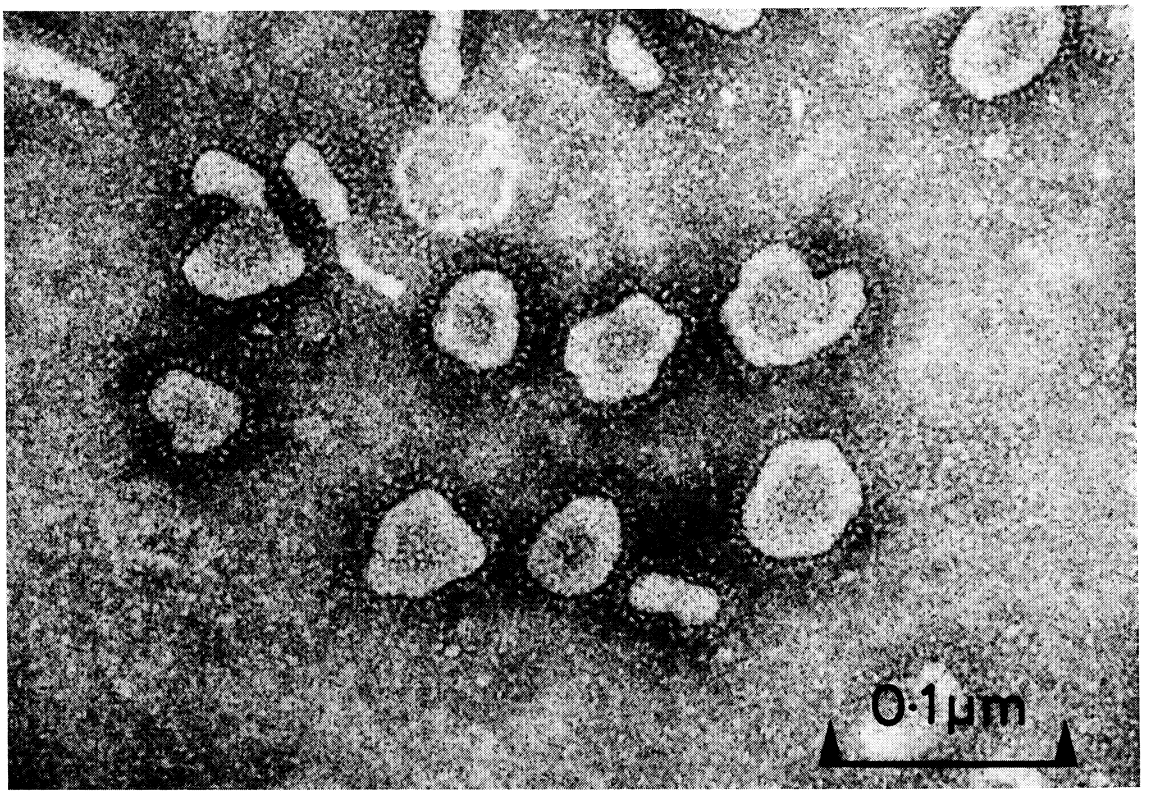

Plate 2B

Reconstituted vesicles of egg phosphatidylcholine and membrane penicillinase from *Bacillus licheniformis*.

The membrane proteins were delipidated and then added to the lipids in octylglucoside. The detergent was removed by dialysis.

Genetics

M Sarvas has produced 70 mutants with defects in penicillinase secretion. (He will join our group for a year in June 1977).

To detect possible precursors of the membrane form of the penicillinase (cf. pre- and proforms of eukaryotic secretory proteins) we will try to synthesize penicillinase *in vitro* by a coupled transcription-translation method. We have received a lambda phage from K Murray (Edinburgh) which contains the penicillinase gene from the *Bacillus licheniformis*.

Methodology

Charge shift electrophoresis

We have developed a simple, rapid and sensitive method to distinguish between hydrophilic and hydrophobic proteins. The proteins are subjected to agarose gel electrophoresis in the presence of a non-ionic detergent (Triton X-100), a mixture of a non-ionic and an anionic detergent (Triton X-100 and sodium deoxycholate), and a mixture of a non-ionic and a cationic detergent (Triton X-100 and cetyl trimethylammonium bromide). The electrophoretic mobility of hydrophilic proteins is unaffected in the three detergent mixtures. However, the mobility of amphiphilic proteins shifts anodally in the Triton X-100 deoxycholate system and cathodally in the Triton X-100/cetyl trimethylammonium bromide system as compared to the mobility in Triton X-100 alone. If specific detection methods are available the proteins to be analyzed need not be pure. In collaboration with F Melchers (Basel) we are now using the method to characterize B-lymphocyte membrane receptors.

Protein micelles

We have previously shown that the E proteins of Semliki Forest virus can be made to form water-soluble lipid- and detergent-free protein complexes containing 8 copies of E_1, E_2 and E_3 (molecular weight 9×10^5). Using the same method, we have now prepared "protein micelles" containing about 20 copies of the amphiphilic membrane penicillinase polypeptide (molecular weight about 6.6×10^5). We have also been able to make mixed micelles of the virus $E_1E_2E_3$ protein and the membrane penicillinase with molecular weights between 9×10^5 and 6.6×10^5 (Plate 3). Electron microscopy of these micelles has been carried out by K Leonard. These studies suggest that hydrophobic interactions play a dominant role in the formation of the protein micelles.

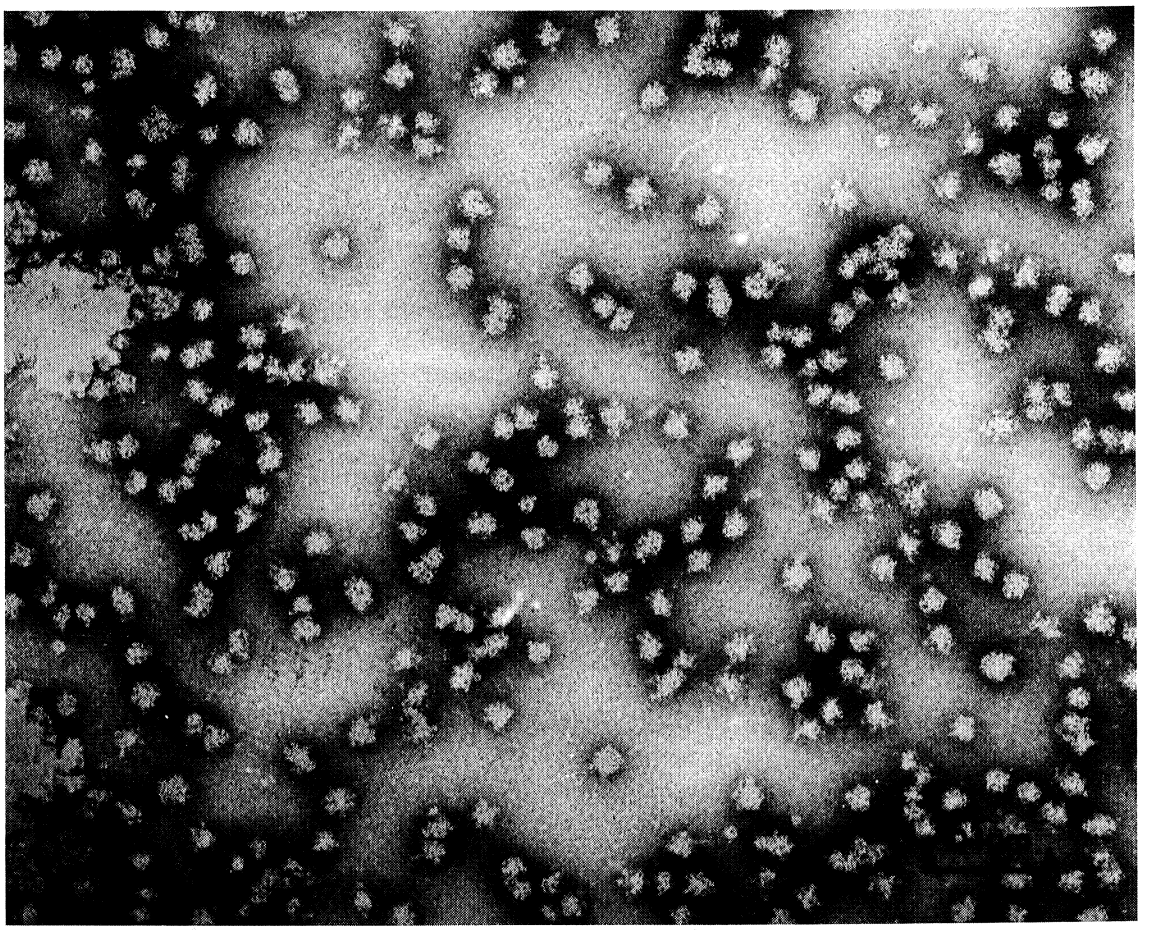

Plate 3

Mixed micelles of Semliki Forest Virus protein and membrane penicillinase (in the ratio 2 : 1).

Chromosome structure and gene regulation

Member: U Plagens

Technical assistants: A d'Arcy*, H Heinz*, P Black*, C Francke* (part-time)

Polytene chromosomes of insects (*Chironomus*, *Drosophila*) have been chosen as objects for studying chromosome structure and gene regulation in eukaryotes. The special features of this system were outlined in last year's annual report; the present account is restricted to a description of the projects and of the progress made.

Project I - Analysis of RNA and proteins of salt-treated polytene chromosomes and nuclear membranes

Studies of proteins extracted from separated chromosomes were terminated at the stage described last year, i.e. one-dimensional gel analysis of the proteins which show characteristic quantitative differences. By using the same methodology, we have since concentrated on analyzing the proteins and RNA remaining after treating isolated chromosomes and nuclear membranes with 2M salt[1,2]. This project was thought to be interesting because although all histones and most non-histone proteins are removed by salt incubation, the polytene chromosomes still exhibit a clear banding pattern, i.e. highly condensed DNA, after staining with ethidium bromide.

The results may be summarized as follows:

1 Salt-treated polytene chromosomes still display a clear banding pattern, the bands forming ring-like rather than disc-like structures.

2 Five non-histone proteins are detectable on SDS gels (MW between 52 and 70,000 daltons) which are also present in the nuclear membrane of polytene cells, in Ehrlich ascites interphase chromosomes, and in DNA isolated by the method of Gross-Bellard *et al.*, (1973)[3].

3 The salt-treated chromosomes are sensitive to DNAase, RNAase, and trypsin treatment; to a much lesser extent to proteinase K, pronase and papain incubation; but not at all to RNAase H. Analysis of the RNA content of salt-treated genomes yields two peaks at 38 s and 25 s.

4 A hypothesis has been developed in which biochemical and cytological findings (regarding chromosome attachment to nuclear membranes in early stages of polytenization) as well as electron-microscopical observations of the fibrous lamina of nuclear membranes[4] have been combined.

These data are being published.

Project II - Distribution of histones and α-amanitin-sensitive RNA polymerase on polytene chromosomes as studied by indirect immunofluorescence

Immunofluorescence studies undertaken in collaboration with A L Greenleaf and E K F Bautz (*Drosophila* RNA polymerase) have been continued and extended to include histone H1 and histone H3 and H4 antisera (in collaboration with S C R Elgin and M Bustin respectively). As described[5], α-amanitin-sensitive RNA polymerase can be specifically located on polytene chromosomes, especially in actively transcribing regions. The double-label technique used shows that histones (H1, H3, H4) cannot be detected in puffs although, when the same preparation is subsequently treated with anti-polymerase serum, the puffs fluoresce as brightly as usual.

The results of the histone-immunofluorescence studies are in process of publication.

Project III - Structural analysis of polytene chromosomes by neutron diffraction

We have modified the methods for polytene chromosome isolation to separate and fix (in formaldehyde) some 6,000 genomes (equivalent to 35 μgr) for neutron diffraction studies. After exchanging the storage media (glycerol : ethanol = 1:1) with deuterated homologues, we intend to compare our results with those obtained from metaphase chromosomes[6]. (This project has been accepted by the ILL, Grenoble.)

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Non-histone proteins

Member: E Jost

Postdoctoral fellow: R W Lennox*

Technical assistant: M Klein*

Research in this group is concentrated
(1) on the superstructure of DNA in chromosomes
(2) on the number and function of the proteins in chromosomes and
(3) on the interaction between DNA and chromosomal proteins.

We are studying certain aspects of the protein composition and arrangement in chromosomes by using affinity chromatography of proteins on various preparations of DNA and on DNA which has been altered by intercalating agents. Specifically, our object is to characterize the nuclear proteins in the erythropoietic cells of chick embryo erythrocyte nuclei undergoing reactivation in heterokaryons.

In cooperation with P R Cook (University of Oxford) we have characterized the proteins which remain bound to nuclei after treatment with high salt concentrations and with detergents. These nuclei sediment through gradients containing intercalating agents in a manner characteristic of DNA that is intact, supercoiled and circular. It was concluded that the DNA is subject to the same kind of topological constraint, restricting the rotation of one strand of the duplex about the other, as that found in circular DNA molecules. Some of the proteins which are still present in nuclei containing superhelical DNA show the same electrophoretic mobilities as proteins from isolated nuclear envelopes. The possible involvement of these proteins in maintaining the superstructure of the DNA is under investigation.

Certain aspects of the protein arrangement in chromosomes are being studied by reassociating proteins with DNA which has been complexed with intercalating agents. It was found that ethidium bromide and actinomycin D when intercalated into DNA prevent some non-histone proteins with high affinity for DNA from binding to the drug-DNA complex. Proflavin and acridine orange, which do not contain bulky side groups, affect the binding of histones, but to a lesser extent that of non-histone proteins with high affinity for DNA. The results can be interpreted in terms of the binding and arrangement of the non-histone proteins along the histone/DNA structure. One class of non-histone proteins seems to bind to DNA by reorganizing the DNA structure in the groove believed to enclose the phenyl and ethyl groups of bound ethidium bromide or the peptidic component of bound actinomycin D.

The presence of histones in a protein/DNA complex reduces the amount of non-histone proteins liberated from protein/DNA complexes by intercalating

agents. This is indirect evidence that the majority of the binding sites for non-histone proteins may be on DNA associated with histones. This hypothesis is being tested further by reassociating non-histone proteins to nucleosomes.

Histones can also be dissociated from DNA by intercalating agents and salt. The effect of various drugs on the dissociation of histones from DNA is being further investigated.

Control of morphogenesis in hydra

Members: H C Schaller, C Grimmelijkhuizen*

Student: T Schmidt

Technical assistant: K Flick

Like an embryo, hydra has two centres of organization, head and foot, from both of which gradients of activation and inhibition extend towards the opposite ends. We are trying to prove that these gradients of biological properties are due to a graded distribution of morphogenetically active substances. So far we have evidence that four such substances exist: an activator and an inhibitor of head formation and an activator and an inhibitor of foot formation. The immediate aim of our research is to isolate and characterize these substances and, as a longer-term project, to study how they interact to control pattern formation in hydra.

Action of the head activator at the cellular level

Steadily growing hydra consist of 5 major cell types: epithelial cells, gland cells and interstitial cells as predominantly proliferating cell types, and nerve cells and nematocytes as differentiation products of interstitial cells. We were able to show that the head activator acts on all these cell types as an unspecific growth-stimulating factor. Under starvation conditions cellular growth is suppressed in hydra; the cells become arrested in the G_2 phase of the cell cycle. If such starved hydra are incubated in head activator, the cells are stimulated to divide. The head activator is thus one of the substances which control cell cycle times and hence growth in hydra.

In addition, the head activator plays a role in the determination of uncommitted stem cells in hydra. Interstitial cells have basically three choices: they can differentiate to nerve cells or to nematocytes, or remain stem cells. To measure the effect of the head activator on interstitial cells it is important to determine when in the cell cycle this determination occurs. We were able to show that the determination of interstitial cells to nerves occurs before or in the very early S period. Head activator influences this determination, if it is present during this time period. In the presence of head activator more interstitial cells are determined to become nerve cells, fewer to nematocytes. The head activator is thus one of the substances which control the determination of cells in hydra.

The head activator is present in hydra in two states, an inactive, structure-bound form and an active, low-molecular-weight form. Circumstantial evidence suggests that under normal steady-state conditions very little of the head activator is available in the free form

and that only during special events are larger quantities released. Thus we have found that, during the first 4 hours after initiation of head regeneration by cutting, head activator is released excessively from the head-regenerating tip. This release is necessary to trigger the events which finally result morphologically in the formation of a head and at the cellular level in the reprogramming of gastric cells to become head-specific cells. One such reprogramming is the determination of interstitial cells to nerves which occurs exactly during the time period when head activator release is highest.

Chemistry

The crux of working with morphogenetic substances is that they are only present in minimal quantities. The head activator is a peptide which consists probably of less than 10 amino acids. If we had enough material, it should be easy to clarify its primary structure. We have tried two approaches, the first being to find sources other than hydra that contain larger quantities of material. One such source is sea anemones which contain a substance that, so far as we can judge, has biological and chemical properties identical to those of the head activator from hydra, and which also contain the other three hydra factors. From the various types of sea anemones available we decided to work with *Anthopleura elegantissima*, because it contains all four factors in sufficient quantities and is readily obtainable at a reasonable price.

The second approach was to label the head activator *in vivo*. Hydra are unsuitable for this purpose, because they do not eat or take up radioactive amino acids easily. Since the head activator, or a very similar peptide, is present in the mammalian brain, we hoped to find cell lines which produce it. Such cell lines exist, but so far none of them produce enough. In the best case head activator amounted to 10^{-7} of the total protein. (This latter project was carried out in cooperation with various other non-EMBL groups.)

Neuronal arrangements which mediate vision in insects

Members: N J Strausfeld, G Geiger*

Technical assistant: M Obermayer

Resolution of neuronal elements

One criterion for recognizing principles of neuronal assembly is to develop reliable procedures which can screen populations of neurons and visualize their specific, rather than general, patterns of distribution. These techniques must also recognize unique cells as being unique rather than giving a single chance visualization of one of many elements.

We are developing two classes of neurostructural procedures; "preparatory" and "reconstitutive". The first class includes methods that will resolve parts, the whole, or multiples of any nerve cell or its components (Plates 4 and 5). The second class includes reconstruction procedures to fit a component into the logical plan of cell assembly (Plate 6).

Although there are many histological techniques for showing up neurons, the great majority are randomly selective or demonstrate features of a fraction of the cell population[1]. These are valuable for resolving some patterns and symmetries within the fibroarchitecture but are impracticable for connectivity studies. Thus we have been concerned with de-randomizing selective procedures and have achieved a modicum of success after empirical tests of electron-microscopical fixations, followed by some radical variations of metal chromate impregnation. The results of these experiments are encouraging: it is now possible consistently to select for and reveal populations of certain types of neurons whose locations meet some of the predicted requirements of retinotopic periodicities[2].

Our results also support the concept that planar assemblies within the mosaic are essentially isomorphic, and predict that isomorphic assemblies are serially arranged through the visual system until the level of the lobula plate[3], the structural interface between lateral expansions of cortex-like optic neuropil and the archaic, ganglionic "brain stem". At this level isomorphic retinotopic mosaics interact with giant or unique neurons (which may also be genetically unique, as are many cells of the ganglionic chain). Both types of neuron provide intermediate channels between cortical neuropil and circuits to motor neuropil. Unique cells (Plate 4) characteristically map only onto specific zones of planar mosaics and thus subserve restricted areas of the visual field. By exploiting the phenomenon of transsynaptic cobalt migration (see the 1975 Annual Report) through specific and contiguous sets of neurons and selective incorporation of cobalt by entire populations of neurons (Plate 6), it is possible to screen identical arrangements in many individuals consistently. Up to the time of writing, four types of unique nerve cell have been reconstructed, in part by using the Cambridge MRC computer graphic facilities (see below). These cells show several interesting structural features with respect to their dendritic branching pattern which may

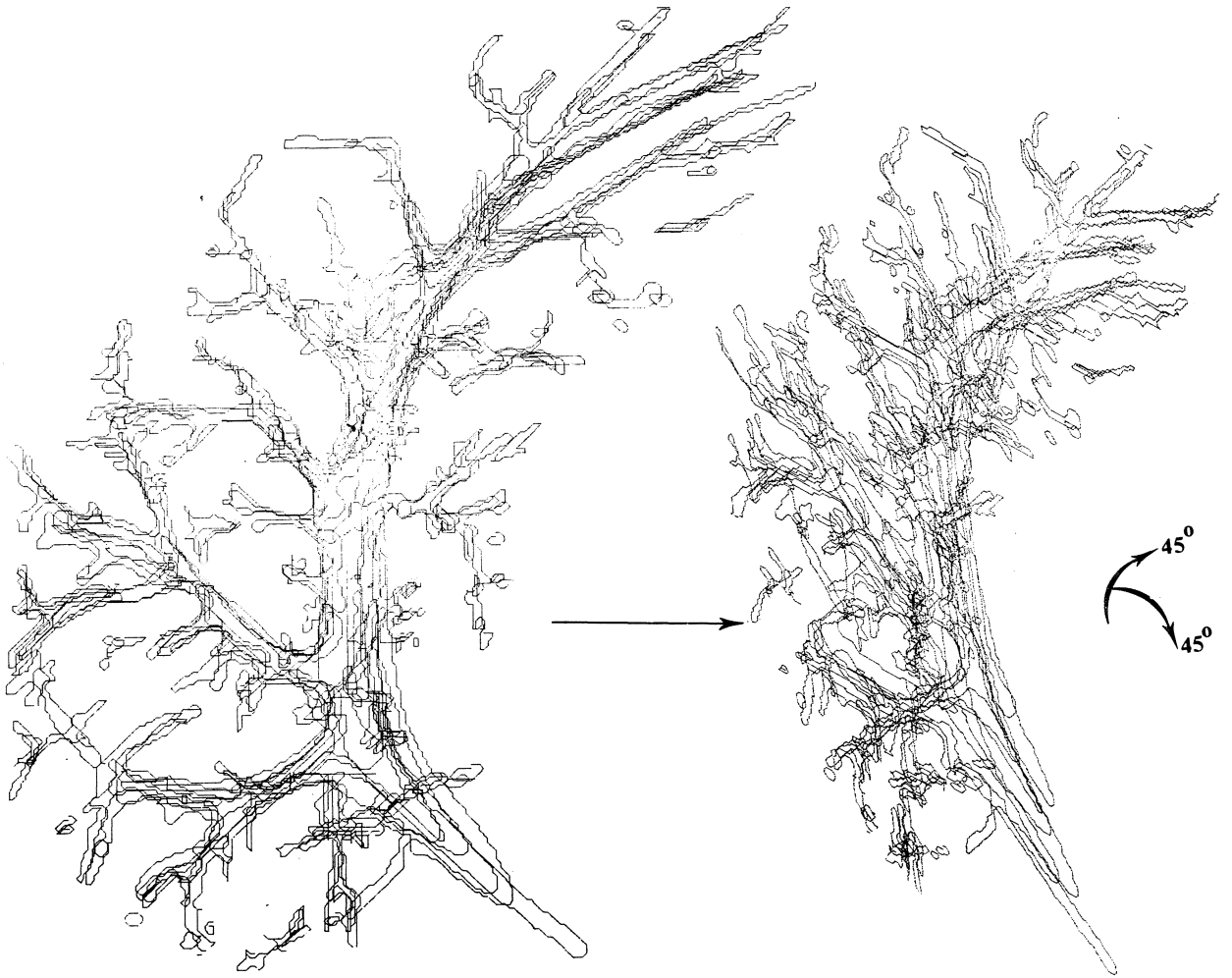

Plate 4

Reconstruction of a unique visual neuron from contours of serial sections. Contour reconstitution was by the neuronal graphics software devised by J White (MRC Laboratory, Cambridge). To the left is shown the original computer reconstruction and to the right the same element after rotation of 45° about the z and y axes.

Plate 5

The unique visual neuron shown in Plate 4 resolved after selective uptake of cobalt chloride injected into the brain. Scale 20 µm.

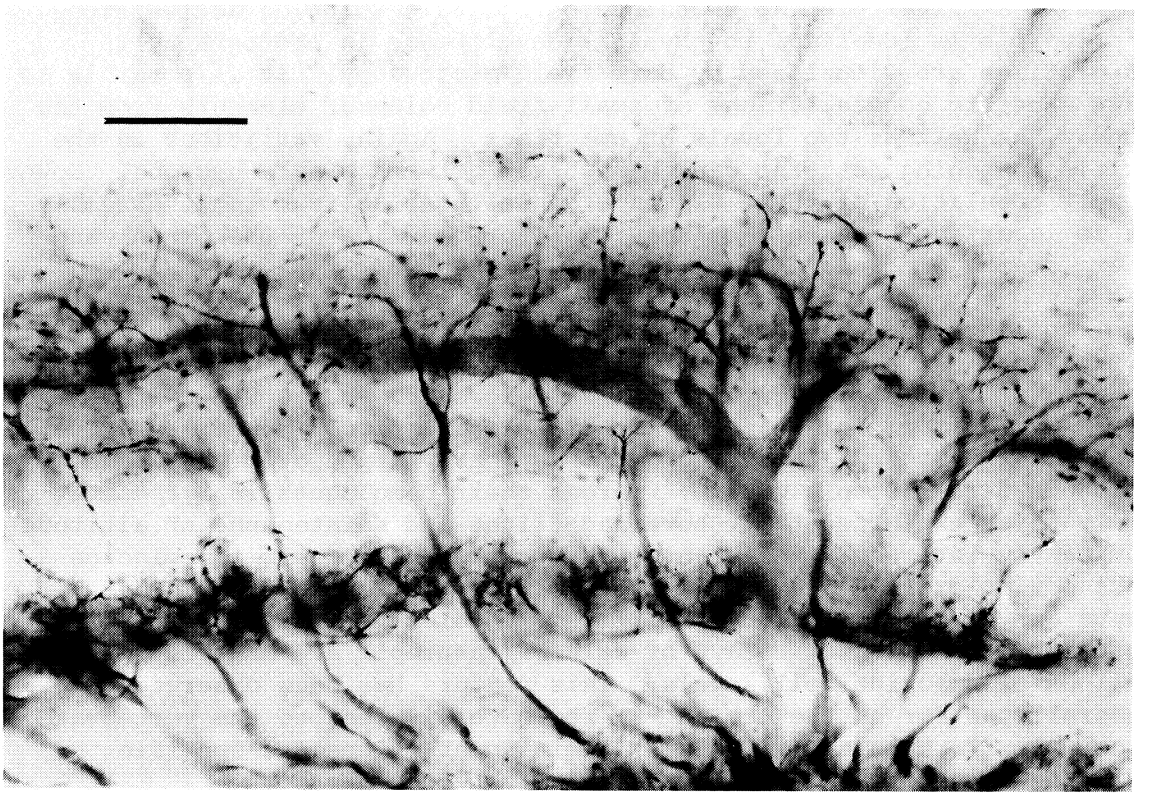

Plate 6

Two sets of columnar neurons, at two levels in the retinotopic mosaic, after selective uptake of cobalt by entire populations of two morphological species of neurons. This type of neuronal display is superior to both the random impregnation methods of Golgi and reduced silver techniques in that it shows up features of neuronal assembly which have been hitherto refractory to other methods. This figure also illustrates the paler profile of the visual unique neuron superimposed upon the upper layer of sub-periodic columnar neurons. Scale 20 μm.

adopt either of two nodal configurations. Both configurations can be present on either side of the brain to impart a left-right asymmetry. However, the actual mapping of their post-synaptic components onto retinotopic input groups is identical between left- and right-hand neuropils. Their domains are thus restricted to invariant boundaries, independent of branching variations. It is this pattern of mapping, rather than the specific geometry of the dendritic tree, as well as the subsequent relationships of neurons with descending "command" elements, that defines its structural rôle within the circuitry. Columnar cells projecting onto unique elements can also be resolved by transsynaptic cobalt diffusion[4], after using a silver plating technique onto cobalt sulphide active sites (the principle is identical to physical development in photography). Their profiles are visualized by dark-field microscopy. It is possible to resolve specific constellations of small-field columnar elements from the periphery, as well as two levels of amacrines. Again, variations in the dendritic branching patterns can be resolved between single neurons. However, the whole population of these elements constitutes an isomorphic assembly which is invariant between one individual (or one side of the brain) and another (or the contralateral lobes).

Comparisons between behaviourally disparate species of *Diptera* reveal that functional systems are essentially identical in terms of projection patterns and dendritic orientations[5,6]. However, the mappings of unique cells differ; for example, the class of vertical motion sensitive elements of *Drosophila*, *Musca* and *Calliphora* are mapped over the whole of the projected retinotopic mosaic, whereas their homologues in *Syrphidae* - a family which exhibits stable hovering flight and maintenance of altitude - predominantly interact with an anterior strip of the mosaic to coincide with binocular overlap. However, peripheral arrangements of columnar elements are isomorphic in both families. Species-specific differences amongst isomorphic planar arrays of columnar neuropil are manifested by variations of amacrine cell levels. This feature has been observed peripherally as far as the lamina - a region equivalent to the vertebrate external plexiform layer[7] - and is reminiscent of species-specific differences of horizontal cell or amacrine cell organization.

Reconstitution of neuronal assemblies

Originally it was envisaged that one procedure for three-dimensional reconstruction could be a combination of holography and microscopy. At present no reflecting condenser system exists which is sufficiently noise-free. An alternative is to take serial photographs of a nerve cell and reconstitute transparencies as a hologram. Pilot experiments, carried out by G Geiger in conjunction with the Physics Department at the University of Hannover, showed that graininess and precise alignment of the photographic series were limiting factors. Only six successive images can be stored free of interference. This gives a three-dimensional image with a maximum depth of 20 μm, and is inadequate for our purposes.

We are now committed to computer graphics. P Speck of the EMBL computer group is collaborating with us in defining algorithms for neuronal analysis and is devising software for the following procedures: three-dimensional

reconstructions of single cells and constellations; rotations; profile averaging; stick diagrams; transformations of distorted hexagonal mosaics to regular rectilinear arrays; mappings of fibres onto rectilinear mosaics; vector analysis of fibre orientations within coordinates natural to planar neuropils. It is envisaged that section reconstitutions and rotations will be operational in July 1977 and that over the coming year software for other procedures will have been devised. Meanwhile we are investigating the practicability of section alignments prior to digitalization, by video-systems as opposed to classical photographic techniques. Until the EMBL graphics system is operational we have recourse to the MRC system in Cambridge, and this has already proved invaluable for resolving mappings and spatial relationships of neurons resolved by transsynaptic diffusions and thin serial sections of material prepared for electron microscopy (Plate 4).

Structure and behaviour

In conjunction with structural studies, one long-term objective is to analyze the development of neuronal assemblies and to draw direct correlates between structure (and its alterations) and behaviour. Instrumentation is being developed (by Geiger) for neuroplast ablation and for deep local ablations in adult neuropil. The device for both types of ablation will consist of a pulse dye laser that can be focussed on the desired cells, or region of cells, by means of simultaneous interference microscopy (for larval ablations) or the superimposition of the head capsule onto a precise atlas of the fibroarchitecture, using video monitoring. All instruments have been ordered or designed and the system should be operational by the summer of 1977.

Behavioural correlates (e.g. ref 8) for the above experiments will be measured by means of a laser torque-transducer coupled to a pattern-generating drum. Both will be linked to a ND 240 computer for on-line analysis. All instruments are at present under construction but will probably not be operational before August 1977.

Dr Gareth Griffiths will join our group in May 1977 and will investigate activity stains for functional constellations of nerve cells. He will also study biochemical aspects of structure, including the distribution of transmitters or their precursors. He will also be involved in electron-microscopical studies of the visual system, including freeze fracture of the postsynaptic membrane.

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Transfer of proteins across membranes

Member: B Dobberstein*

Biological membranes present a diffusion barrier to macromolecules such as proteins. However a large number of specific proteins have to cross a membrane to reach their place of destination and function. Segregation by a membrane is required not only for secretory proteins but also for lysosomal and peroxysomal proteins, for certain mitochondrial or chloroplast proteins synthesized in the cytoplasm, and for some bacterial and plant toxins.

The discovery of membrane-bound ribosomes, and the demonstration that nascent chains synthesized on membrane-bound ribosomes are vectorially discharged across the membrane, suggested that the ribosome-membrane junction may function in the transfer of proteins across a membrane. However, this junction did not explain how the cell determines which proteins can traverse *via* the ribosome-membrane junction. In the recently formulated signal hypothesis, it has been proposed that an amino-terminal extension ("signal sequence"), common to all nascent secretory proteins, provides coordinated binding of the ribosome to the membrane and the cotranslational transfer of the nascent chain into the intracisternal space of the endoplasmic reticulum. The transfer is accompanied by the removal, which at least for some secretory proteins is cotranslational, of the signal sequence by a membrane-bound signal peptidase.

Work begun in G Blobel's Laboratory at the Rockefeller University on the characterization of the signal peptidase, and of a postulated membrane receptor for signal sequences and the large ribosomal subunit, will be continued.

In the light of the signal hypothesis it was interesting to see whether proteins transferred from the cytoplasm into a cell organelle are synthesized on free or membrane-bound ribosomes and as higher molecular weight precursors. The small subunit of the chloroplast enzyme ribulose-1,5-biphosphate carboxylase is synthesized in the cytoplasm and transferred into the chloroplast, where it combines with the large subunit to form the holoenzyme. We were able to show[1] that the small subunit is synthesized in a cell-free system from wheat germ as a higher molecular weight precursor which can be proteolytically processed to the authentic small subunit and a small fragment. A function of the precursor in membrane transfer has been proposed. Our results further show that the small subunit is synthesized on free ribosomes. Thus the ribosome-membrane junction and cotranslational transfer are not required for all cytoplasmic proteins which are transferred across a membrane.

Transfer of completed proteins across membranes is known to take place for some bacterial toxins such as diphtheria or cholera toxin and also for the plant toxins abrin and ricin.

Work is in progress to establish the location - amino or carboxyl terminal - and the amino-acid sequence of the additional amino-acid residues in the precursor of the small subunit of ribulose-1,5-biphosphate carboxylase. Further it will be necessary to isolate, characterize and localize the protease which converts the precursor into authentic small subunits. Reconstitution of a functional *in vitro* system capable of transferring the precursor of the small subunit into a chloroplast is planned.

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DIVISION OF BIOLOGICAL STRUCTURES

Cytochrome b from the mitochondria of *Neurospora crassa*

Member: H Weiss

Postdoctoral fellows: B Ziganke, E Herz*

Visiting worker: K Müller

Technical assistants: B Juchs, A Krebs

Objectives

The energy of eukaryotic cells is predominantly supplied by the oxidative phosphorylation system of mitochondria. This is a complex chain of chemical reactions mediated by a large number of proteins of the mitochondrial membrane. These reactions can be subdivided into oxidation-reduction reactions which generate intermediate energy, and ATP-synthesizing reactions which trap the intermediate energy and conserve it in the form of the energy-rich compound ATP. Although many hypotheses have been proposed, the molecular basis of the generation and transfer of the intermediate energy is still unknown, probably owing to our lack of information about the structure of the components involved.

The objectives of the group are therefore directed towards improving methods for the isolation of membrane components that catalyse the oxidation-reduction reactions and for studying their structures and mode of assembly.

Results

We have so far obtained the following cytochrome b-containing preparations of increasing complexity as monodisperse solutions, in the presence of various detergents.

a) Cytochrome b a dimeric haem protein (M_r 55,000) consisting of two haem-binding subunits (α and β), which are at this stage of isolation indistinguishable with respect to the properties of the two haem groups (light absorption spectra and oxidation-reduction potentials).

b) Cytochrome b, c_1-complex This is a preparation (M_r 120,000) which consists of dimeric cytochrome b, cytochrome c_1 (M_r 30,000), and three additional polypeptides (M_r 8,000 - 14,000) without prosthetic groups. In this preparation cytochrome b appears to be in its natural environment because the properties of the two haem groups closely resemble those of the two b-type cytochromes observed in intact mitochondria.

c) Cytochrome b, c_1, c-complex This preparation contains in addition one molecule of cytochrome c which is bound with a dissociation constant of 0.1 μM.

d) Cytochrome b, c_1, c, (Fe-S)-complex a preparation (M_r about 200,000) which contains in addition one polypeptide (M_r 20,000) carrying an iron-sulfur group and two polypeptides (M_r 45,000 and 50,000) without prosthetic groups. It seems to be the smallest membrane component which is able to mediate electron transport from reduced ubiquinone to cytochrome c.

We have now begun to study the arrangement of the single polypeptides in this last membrane component by means of intermolecular cross-linking of the polypeptides prior to dissociation of the preparations, and by means of surface labelling of the two membrane faces prior to the isolation of the preparation. For these studies the mitochondria of *Neurospora crassa* prove to be especially appropriate owing to the fact that several components of the preparation can be selectively labelled: the mitochondrially translated cytochrome b by *in vivo* incorporation of radioactive amino acids after inhibition of the extramitochondrial translation system; cytochrome c_1 by *in vivo* incorporation of radioactive δ-aminolaevulinic acid into the covalently-linked haem group; and cytochrome c by binding a cytochrome c preparation purified from radioactively-labelled cells.

Results obtained so far show that the cytochrome b subunits are embedded in the hydrophobic interior of the membrane, whereas the other components are oriented towards one of the two hydrophilic faces of the membrane. Cytochrome c_1 seems to be arranged between one cytochrome b subunit and cytochrome c on the outer face. The 14,000 M_r polypeptide appears to interact with the other cytochrome b subunit on the inner face of the membrane. We hope to solve the complete puzzle of localising all the parts of this membrane component in the near future.

Electron microscopy of nucleic acids

Member: H Delius

Technical assistant: M-T Sagne

Electron microscopical measurements have become a very important tool in nucleic acid research. The spreading of the filamentous molecules within a two-dimensional surface film of cytochrome c (Kleinschmidt technqiue) allows direct visualization and length determination of nucleic acids. The technique has found widespread use in many laboratories. The following examples from H Delius' earlier work at the Cold Spring Harbor Laboratory may serve to illustrate some of its many possible applications:

Length measurements and molecular weight determinations of phage and viral DNAs, DNA fragments produced by restriction enzymes, and of viral RNAs [1,2,3,4].

Visualization of the structure of folded bacterial chromosomes[5,6].

Analysis of the complexes between DNA and DNA binding proteins[7,8,9].

Analysis of replicative structures of phage DNA[10,11].

Analysis of transcription complexes of phage and viral DNA with *E. coli* RNA polymerase[12,13,14].

Characterization of restriction fragments by partial denaturation mapping in order to determine their position on the intact genome[15].

It will be the aim of the group to make these techniques available for collaborative projects; to develop technical improvements; and to further computerize the measuring procedures.

Research projects

a) In collaboration with G Bornkamm and B Fleckenstein (Erlangen) partial denaturation patterns of different types of Herpes and Epstein-Barr virus DNAs were analyzed. A new technique is being developed to partially denature specifically those regions with high GC base content, using glyoxal instead of formaldehyde. *Herpes saimiri* and *H. ateles* have terminal stretches of repetitive high GC-containing DNA. By preferentially denaturing these regions it is possible to determine the size of the non-denatured unique virus genome and the extent of unique sequences in terminal restriction fragments.

b) A project has been started in collaboration with J Besemer (Köln) to carry out a heteroduplex analysis of Ø80 phage DNA carrying insertion sequences (which exert promoter or anti-promoter activity). Revertants occur by deletion of these elements, and the extent and position of the deletions will be mapped.

c) An analysis of the transcription of T5 phage DNA by *E. coli* RNA polymerase is being carried out in collaboration with D Stüber and H Bujard (Heidelberg). *In vitro* synthesized RNA is complexed with T4 gene 32 protein, in order to extend it for length measurements while it remains attached to the DNA template (Plate 7). The measurement of number, length and position of RNA chains permits the determination of promoter positions, initiation rates, and direction and rate of RNA synthesis. These findings will be correlated with results obtained by chemical means in Bujard's laboratory.

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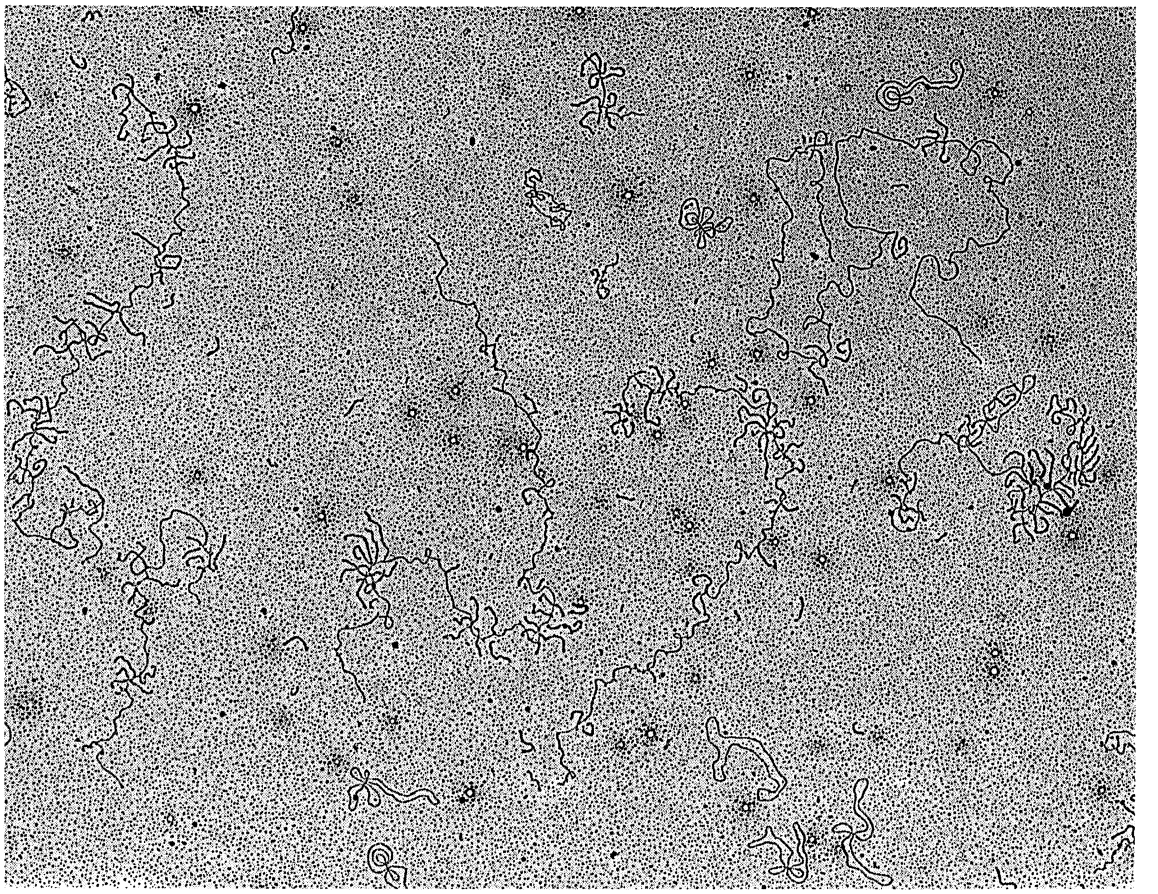

Plate 7

A mixture of Eco RI restriction fragments of T5 DNA was transcribed *in vitro* for 2 min at 37°C by *E. coli* RNA polymerase. T4 gene 32 protein was attached to the RNA chains. After fixation with glutaraldehyde the sample was prepared for electron microscopy by cytochrome spreading, staining with uranyl acetate and rotary shadowing with platinum. Circular molecules are PM2 DNA added as internal length standard.

High resolution electron microscopy

Member: K Leonard

Technical assistant: T Arad*

The aim of this group is to obtain, by both conventional transmission electron microscopy (CTEM) and scanning transmission electron microscopy (STEM), structural information about biological macromolecules and macromolecular assemblies at the highest possible resolution. The present resolution limit for most biological material (approximately 1.5 to 2.0 nanometres) is several times higher than the theoretical limit for the electron microscope itself, a consequence of the effects of specimen preparation and electron beam damage. These effects must be minimized in order to obtain a significant improvement in resolution.

A Philips EM400 CTEM was installed in May 1976; it is fitted with a eucentric goniometer stage which enables the specimen to be tilted over a range of $\pm$ 60°, and also permits the use of a number of special specimen holders. The instrument has performed well over the first months of installation and is being updated as new features become available. The work carried out has included internal collaboration with the K Simons - A Helenius group on the structure of solubilized viral membrane protein aggregates and on reconstituted lipoprotein vesicles (see Plate 2) and with the groups of H Weiss and E Jost. Collaborative work is also being continued with L N Johnson (Zoology Department, Oxford) on phosphorylase b and with K Reid (Biochemistry Department, Oxford) on complement proteins.

Installation of the STEM is described elsewhere in this report. Work on the STEM to date has been concentrated on the use of test specimens to investigate the optimum imaging conditions in bright field and dark field and to evaluate the possibilities of electronic contrast enhancement (see Plate 8). In the latter context, preliminary work done in collaboration with the Oxford ARC muscle group and with the groups of K C Holmes and W Forssmann in Heidelberg has indicated that good images can be obtained for very lightly stained or unstained thin sections. Although use of the STEM for true 'low-dose' imaging will require completion of the microscope computer interface, scheduled for the first half of 1977, considerable progress has been made using 'one-frame' exposure methods to reduce electron beam damage and specimen contamination in the normal operation modes.

Specimen preparation facilities and darkrooms for film development and printing have been set up adjacent to the STEM and CTEM. Work is being carried out on methods of reducing the high contamination rate of biological specimens when observed under normal imaging conditions in the STEM. An ultra-high vacuum evaporator being developed by the Instrumentation Division will facilitate the preparation of clean, contamination-free support films. Emphasis is also being given to low temperature methods for the preparation and examination of specimens in order to reduce damage caused by dehydration and irradiation.

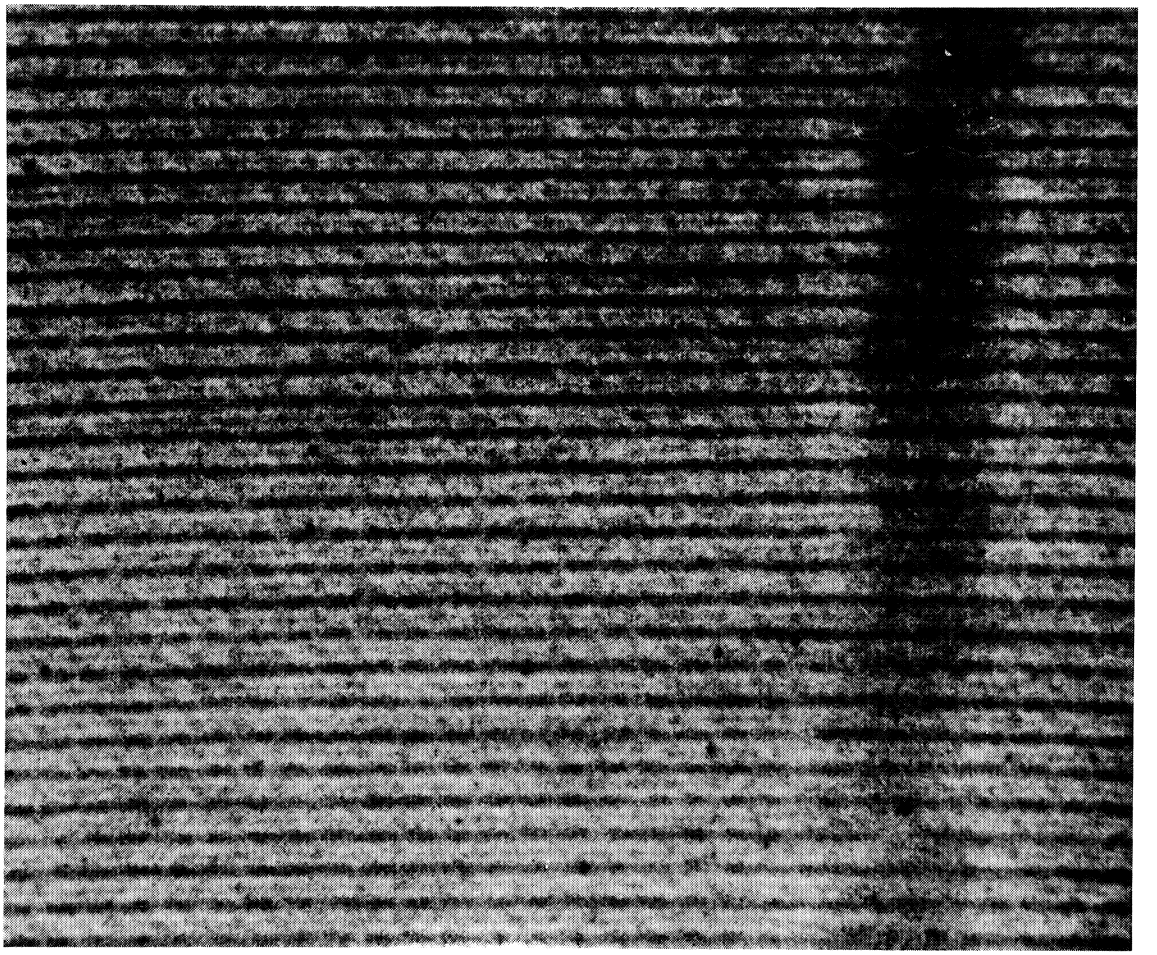

Plate 8

Unstained section of insect flight muscle taken with the STEM, contrast enhanced by mixed signal imaging (for details see text). Thick and thin filaments and cross-bridges are clearly visible (x 160,000).
A Jones and K Leonard

A folded optical diffractometer has been built for the analysis of diffraction patterns from micrographs of periodic structures, and for surveying images prior to computer analysis. The diffractometer can also be used for optical filtering of images, but it is envisaged that this will mainly be done by computer image analysis facilities which are at present being established in collaboration with the computer group.

Structure and assembly of filamentous bacterial viruses

Members: D A Marvin*, J E Ladner*

Technical assistants: S Fowler*, H Siegrist*, H Kabsch* (part-time)
F J Marvin*(part-time)

What is the design of complex molecular assemblies (for example chromatin, muscle, neurofilaments, membranes) and how is this design expressed in function? Our approach to this question has been to select as a model a minimal system that nevertheless carries out important biological functions, and to study this system intensively from many different standpoints, with a view to discovering principles of design and function that will be universally applicable. For this purpose we have chosen the filamentous bacterial virus system. Bacterial viruses are useful for this kind of study because they are available in large amounts, because they are readily accessible to genetic manipulation, and because they grow in a well-defined host. Filamentous bacterial viruses are especially interesting both for their unusual life-cycle and for the relative ease with which several intermediate stages in the life-cycle can be studied. The virus itself is a rod-like nucleoprotein comprising a DNA core encapsulated in a coat of several thousand helically arranged protein molecules. Assembly of this virus structure starts with pre-packaging of the DNA by an assembly protein (not the final coat protein) in parallel with a pre-placement of the viral coat protein in the cell membrane. The act of assembly involves displacement of the assembly protein from the DNA by the coat protein as the completed virus is extruded through the cell membrane. Our immediate goal is to use x-ray diffraction techniques to determine the molecular structures of the DNA/assembly protein complex, of the membrane/coat protein complex, and of the completed virus.

In our first months at EMBL we have been involved in setting up x-ray equipment, installing computer programs on local computers, and teaching techniques to new staff. However, we have learned a few new facts about the structures under current study.

DNA/assembly protein complex We have implemented published procedures for preparing purified assembly protein and DNA/assembly protein complexes. Samples for protein crystallization have been laid down, and preliminary x-ray fibre patterns of the DNA/protein complex have been obtained.

Membrane/coat protein complex Published procedures for reconstitution of coat protein in the bilayer of phospholipid vesicles have been tried in collaboration with A Helenius, with promising results. This project is temporarily quiescent owing to lack of staff.

Virus structure Most of our effort has been applied to further analysis of the virus structure by x-ray diffraction. We have surveyed the interaction of a number of heavy atom compounds with the virus, in the hope

of using heavy atom derivatives to resolve a controversy about whether the number of proteins in one turn of the virus helix is 4.4 or 5.4. We have not yet obtained a heavy atom derivative giving clear intensity changes on the diffraction pattern, but one of the samples gave a pattern that is among the best yet obtained. (Plate 9).

At the same time we have been attempting to identify the conditions necessary to cause the structural transition between the two forms of the virus with 4.4 and 4.5 (or 5.4 and 5.5) units per turn respectively. None of the conditions tried so far (ionic strength, divalent cations, pH) have any definable effect on the transition. However, in the course of the survey we discovered that the lithium salt gives an unusual diffraction pattern, indicating that some structural changes are induced by the presence of lithium.

We have also been collaborating with R Ladner of the Computer Group in developing computer graphics as a means of displaying and manipulating trial models.

Plans for the future

During the first part of 1977 we intend to concentrate on the structure of the virus. We shall continue refining present models, including as constraints both the fit of the calculated transform to the observed diffraction pattern and stereochemical information on local structure. We shall attempt to resolve the 4.4 *versus* 5.4 controversy by further search for heavy atom derivatives and by attempting to build feasible models of each structure. The best models will be tested for consistency with the 4.4 to 4.5 transition, since only models that are consistent with this transition are acceptable. We also hope to develop more fully some of our ideas on the applicability of the principles of filamentous virus design to other structures such as myosin or neurofilaments.

Later in the year more of our effort will go towards study of the DNA/assembly protein complex. We have initiated a collaboration with the Outstation at Grenoble on a neutron diffraction study of this complex, with the aim of using density matching to define the relative positions of DNA and protein in the complex. We also hope to look much more extensively at the structure using the techniques of electron microscopy (in collaboration with H Delius) and x-ray fibre diffraction.

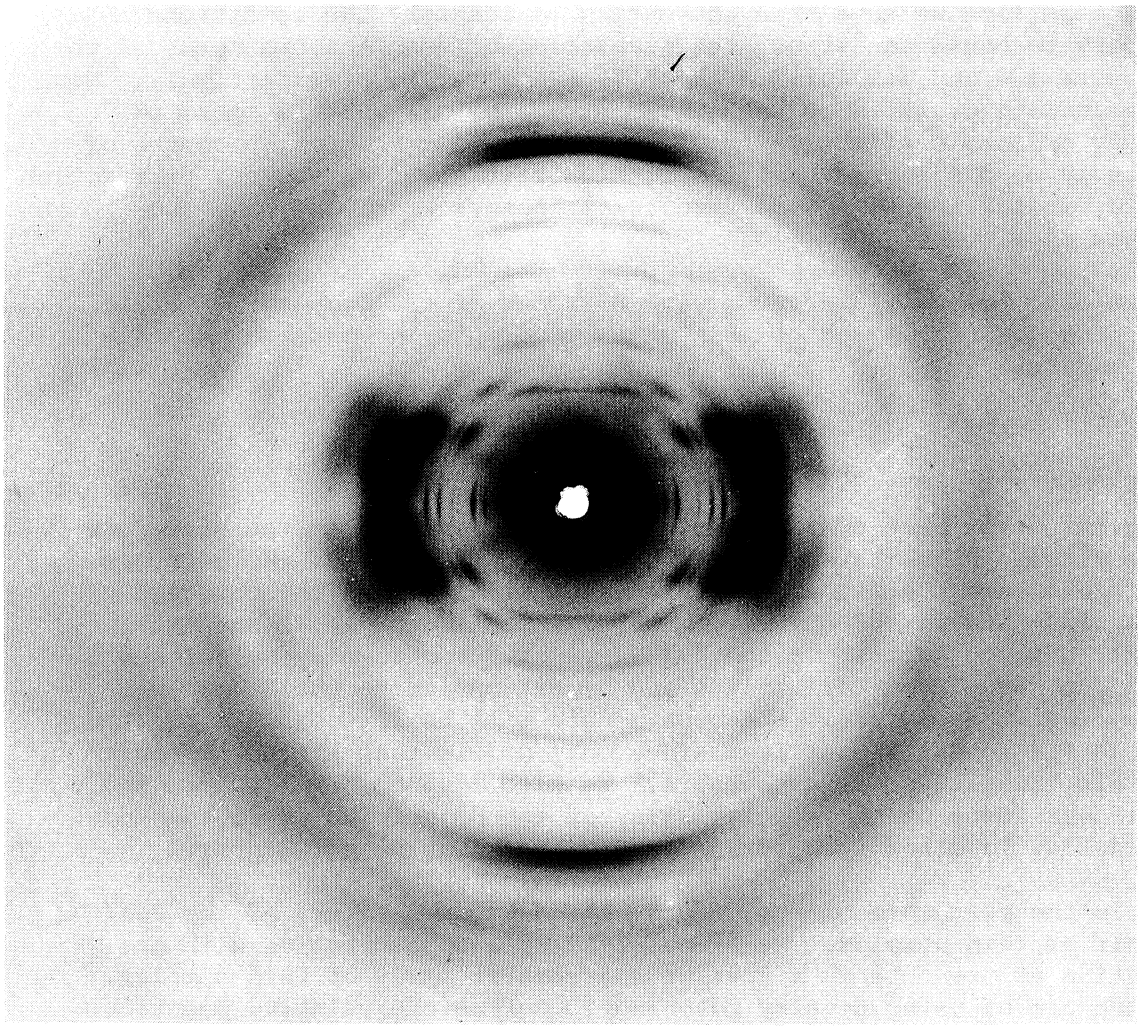

Plate 9

X-ray fibre diffraction pattern of an I_2 derivative of the Pf1 strain of filamentous bacterial virus (from W Königsberg). Specimen-film distance 7.3 cm, enlarged x 2. (Fibre G6F, film 2217).

Bacterial polypeptide elongation factors

Member: R Leberman*

Student: W H Gast*

These studies are being carried out in collaboration with the following staff of the Max-Planck-Institut für medizinische Forschung in Heidelberg

Scientists: W Kabsch, G E Schulz, A Wittinghofer

Students: Z Acosta, R Frank

Technical staff: R Giovanelli, G Helmig

Our aims are to study one particular step of the process of protein synthesis in bacteria, namely the elongation step, at the atomic level. In order to do this, we hope to be able to crystallize various components involved in forms suitable for x-ray diffraction analysis. Various protein factors take part in the elongation step, and we have succeeded so far in growing large crystals of one functionally important factor (EF-Tu) and also of a large fragment of it. These crystals are suitable for x-ray diffraction studies and data are being collected from them.

Using inorganic salts or organic solvents as precipitants, we have been unable to obtain crystals of the elongation factor EF-Tu (isolated from either *Escherichia coli* or *Bacillus stearothermophilus*) suitable for x-ray studies[1], whereas in another laboratory pseudotetragonal crystals of the complex between EF-Tu and GDP were grown using organic solvents[2]. However, using polyethylene glycol we were able to find conditions to grow suitable crystals within 3 weeks (Plate 10). These crystals exhibited a large degree of polymorphism[3], all the crystal forms being related and belonging to either trigonal or hexagonal space groups. Occasionally pseudotetragonal crystals were obtained but we were able to demonstrate that the protein in these crystals was degraded (probably enzymatically), and further that it was possible to produce this type of crystal by pretreatment of EF-Tu with trypsin[4]. Recently we have crystallized a large (M.W. 39,000) tryptic fragment of EF-Tu[5] which has many of the biological properties of the native protein (Plate 11)[6,7]. The crystals belong to an orthorhombic space group; they diffract very well; they are stable; and one isomorphous heavy atom derivative is already available. Data collection is well under way and by the time this report is distributed we hope to have a 6 Å Fourier map.

We have also obtained small crystals of the complex formed between the two elongation factors EF-Tu and EF-Ts. The crystals are of two types - one of the complex formed by the two factors from *E. coli*, and the other of the complex EF-Tu from *E. coli* with EF-Ts from *B. stearothermophilus*. The crystals are at the moment too small for x-ray diffraction studies but

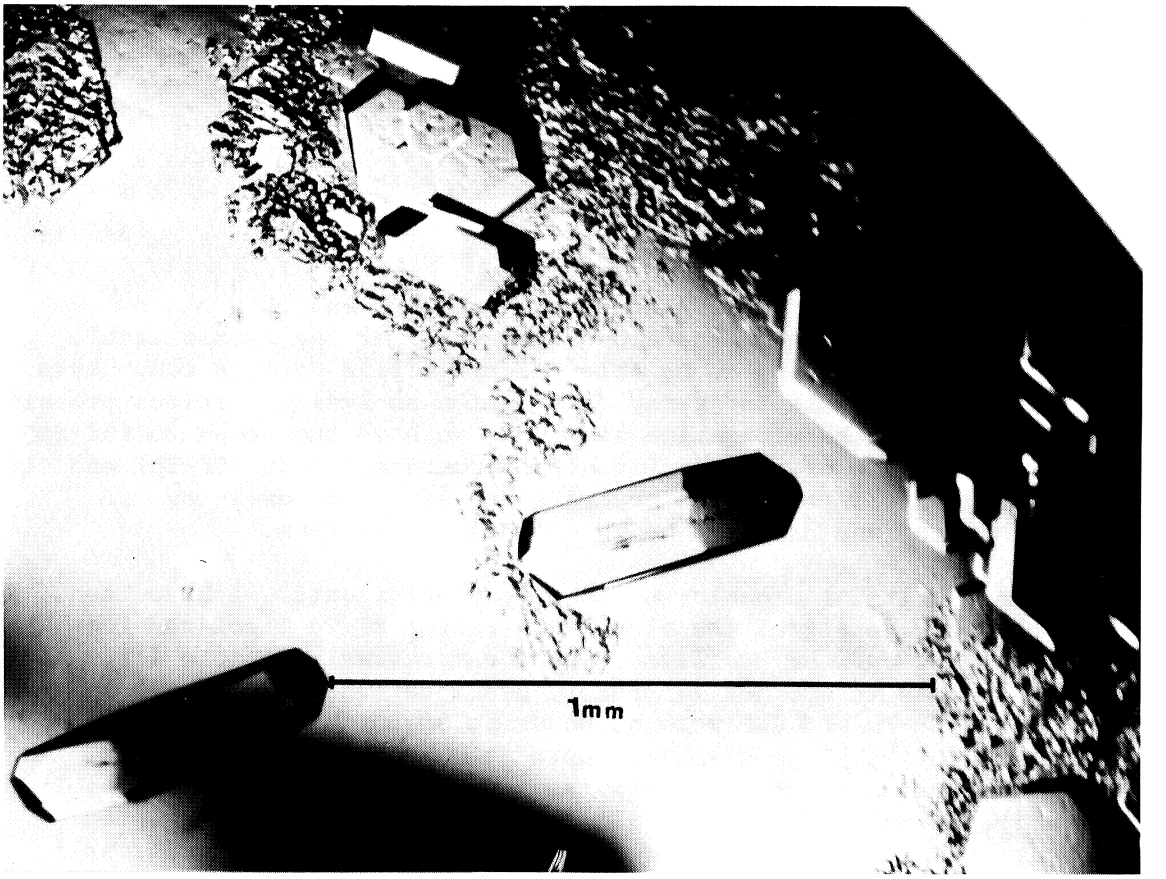

Plate 10

Crystals of native EF-Tu.GDP complex, from polyethylene glycol 6000 solution.

Plate 11

Crystals of a large tryptic fragment of EF-Tu (Fragment A) complexed with GDP, from polyethylene glycol 6000 solution.

we do have approximate conditions for growing them. One problem here is the small quantities of EF-Ts that can be isolated from bacteria.

In order to find starting parameters for attempts to crystallize the ternary complex between EF-Tu, GTP, and aminoacyl-tRNA, the stabilization of various aminoacylated tRNAs by EF-Tu.GTP has been studied under various solvent conditions. Relatively large amounts of one species of aminoacylated tRNA are at present being prepared for attempts to crystallize the ternary complex.

The structural requirements for the binding of GDP by EF-Tu have been investigated using a number of natural and synthetic nucleoside diphosphates as competitors. It was found that the oxygen in the 6-position of the base moiety was more important than the amino groups at the 2-position.

We have also looked at the divalent metal ion requirement for the binding of GDP to EF-Tu. Our results indicate that GDP is bound only as a metal complex, with Mn^{2+} able to replace Mg^{2+} very effectively. At higher concentrations Co^{2+} and Ni^{2+} are also effective.

In the past few years interest in EF-Tu and EF-Ts has increased. This has been due not only to their roles in protein biosynthesis, but also to their participation in other cellular processes. For example, the factors are subunits of Qß-replicase, and EF-Tu has been implicated in the control of DNA-dependent RNA synthesis in uninfected cells. Thus it may be that the structures of these factors and their complexes will be useful in elucidating the mechanisms of a variety of biological reactions.

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DIVISION OF INSTRUMENTATION

STEM development

Members: A V Jones, J-C Homo*, B M Unitt*

The basic HB5 STEM was delivered in May 1976. After a period of testing with a variety of specimens, the initial instrument development concentrated on two aspects:

(1) the development of instrumentation and operating procedures to minimize electron beam irradiation under normal operating conditions;

(2) the use of analogue video processing to improve image contrast on difficult specimens, e.g. unstained specimens which are generally not imageable in a conventional transmission electron microscope.

The central requirement was the provision of some form of image storage so that the irradiating electron beam could be switched off during prolonged periods of observation. The storage facility would also be used to compare processed and unprocessed images to determine whether or not the processing had improved the image quality. This processing may be analogue (as described here), or by computer (scheduled for initial development in 1977).

Analogue video processing takes two forms. In the first form, the signal from a single detector system is electronically modified to enhance certain features of the image. For example, differentiation increases the contrast of edge detail and can be used to produce a pseudo-3-dimensional image as in Plate 12. Alternatively (or additionally) the signals from several detectors can be combined in various ways to produce enhanced contrast, improved signal/noise ratio etc.

Image storage and minimum-dose operation

The HB5 is basically a two-channel instrument, i.e. it is designed to display simultaneously two independent video signals selected from the available detector systems or from the image storage facilities. Both of the two video channels have now been fitted with a "scan-converter" analogue storage system, each of them being capable of storing sequentially up to 4 separate images.

The scan convertor control unit provides for separate control of the "Erase", "Store" and "Display" functions together with an automatic sequence which erases the previous image, stores the new image at a pre-selected rate and finally displays the image at T/V rate to provide flicker-free images of a quality consistent with the slow storage rate. The unit provides control signals to switch on the microscope beam only during storage.

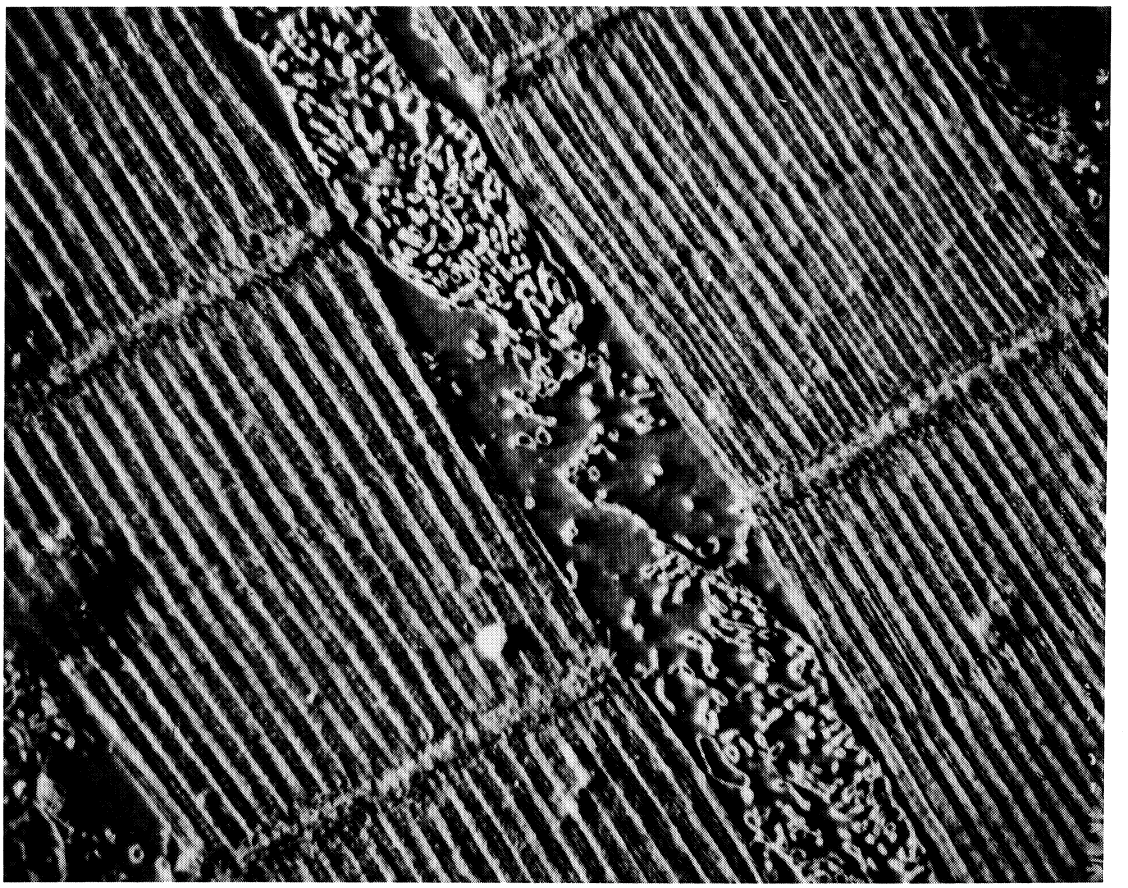


Plate 12

Pseudo-3-dimensional image of sectioned insect flight muscle, Pb-stained, obtained by analogue differentiation x 20,000.

A "Transfer" facility enables a stored image to be re-recorded on the other scan converter *via* the video processor. By this means the correct processing parameters for subsequent direct photographic recording can be predetermined without irradiating the specimen.

The photographic recording unit itself has been modified to permit alternative use of Polaroid or 70 mm roll-film with automatic film advance and shutter operation.

The scan convertor and camera have been linked to the normal microscope controls to simplify the overall operating procedures. Those procedures will be modified in the light of experience to provide a set of automated operations appropriate to biological specimens.

Analogue video processing

As delivered, the STEM was fitted with two detectors corresponding to the "dark-field" and "energy-filtered bright-field" imaging modes of a conventional transmission electron microscope together with a 3-channel analogue mixing unit capable of adding, subtracting or ratioing up to 3 separate signals.

To those facilities have now been added:

(1) an additional non-filtered "bright-field" detector;

(2) additional video input channels for this detector, an x-ray detector and an additional (non-specified) photomultiplier detector;

(3) video switching capable of handling up to 9 inputs (including read-out from the scan converters and from the computer which will shortly be interfaced to the STEM);

(4) a dual channel video processor providing the following functions

(a) automatic video bandwidth limiting to optimize the signal/noise ratio for a particular scan rate;

(b) variable contrast correction to compensate for excessive image brightness variations and thereby to utilize fully the gray-scale range of the recording systems;

(c) controlled differentiation of the video signal to increase the contrast of edge detail in the image;

(5) a wide-band beam current detector/amplifier, used in conjunction with the analogue mixer to suppress image noise caused by beam current variations.

These additions have only recently been completed and are currently being used to study unstained muscle sections (with K Leonard (EMBL), W Forssmann (Anatomy Department, University of Heidelberg), and W Hoffmann (MPI für

medizinische Forschung)) and unstained DNA (with K Leonard and H Delius (EMBL)). The latter study will be extended to include DNA selectively stained with heavy atoms (with H-P Vosberg (MPI für medizinische Forschung), F Eckstein (MPI für experimentelle Medizin, Göttingen) and H Bujard (University of Heidelberg)).

Detector systems for ionizing radiation

Member: A Gabriel

Technical assistants: J-M Dubois*, F Dauvergne*

For many x-ray scattering experiments, especially with unstable biological materials, exposure time is a critical factor. One way of reducing exposure times is to increase the beam intensity either by using rotating-anode tubes or, more recently, synchrotron radiation. Another method is to use position-sensitive proportional counters instead of photographic film. On film 10^6 x-ray photons/mm^2 give an optical density of 0.1, the minimum needed to exceed the noise level; thus the intensity of a beam with cross-section 0.2 x 5 mm can be measured with a precision of 3% if 10^5 photons are incident. With a position-sensitive detector no signal is recorded if no x-rays are incident, so the noise level is zero, and hence the corresponding precision is 0.3%. Thus for the same statistical error the counting time can be reduced by a factor of 100. In addition the proportional counter has a much greater dynamic range than film.

Linear detector

The counter is shown in Plate 13. The anode, a gilded tungsten wire of diameter 10 pm, is maintained at high voltage, while the cathode on both sides of the anode is at earth potential. The geometry of the cathode permits the localization of the ionizing event; it consists of a cell of plexiglass (or fibre-glass) the lower surface of which is covered with parallel conducting strips produced by the evaporation of aluminium. X-rays enter through a beryllium window. The strips are connected to a delay line divided into as many elements as there are strips; this delay line, from the point of view of the electronic circuit, constitutes an image of the cathode. The cell is filled with gas, e.g. argon/methane or argon/CO_2, at 2 atm pressure.

As in all proportional counters there is a capacitative coupling between anode and cathode; thus a build-up of charge is induced on the cathode when a signal arrives at the anode. The distribution of charge on the cathode elements will be influenced by the arrival of an electrical pulse anywhere on the anode, the influence on a given cathode element depending on the solid angle subtended by the element at the point of arrival of the photon. The charges along the line will flow in both directions, giving rise to two electric signals, and a comparison of the time dependence of these signals permits a measurement of the respective distances they have travelled and gives the position of the ionizing event. It should be noted that the localization is not limited by the spacing of the strips and is continuous along the detector.

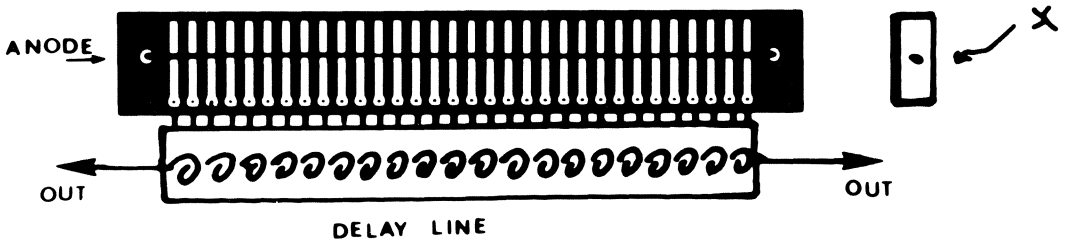


Plate 13

Sketch of a one-dimensional counter (for description see text)

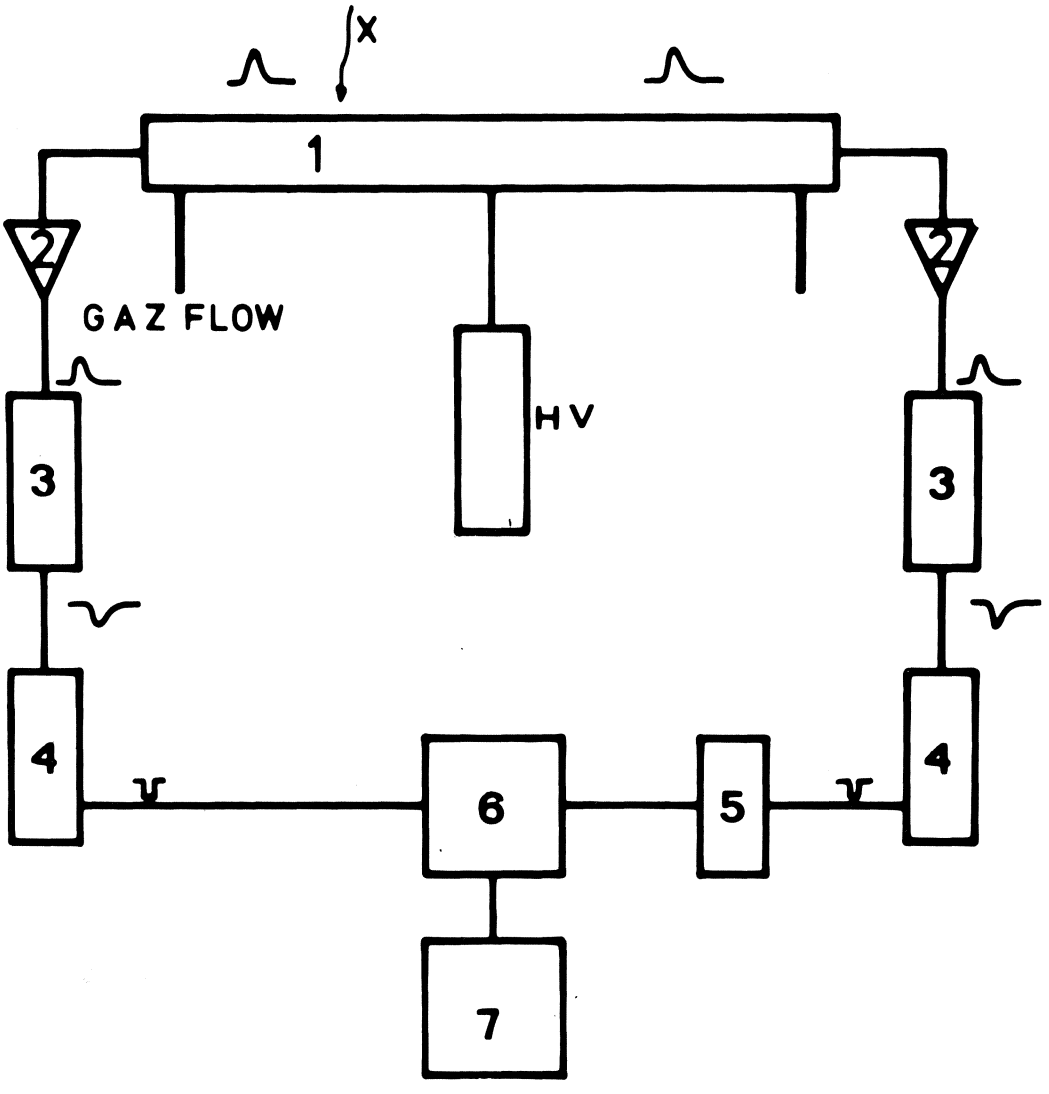


Plate 14

Sketch of complete one-dimensional detector system (for description see text).

Plate 14 shows the design of the one-dimensional detector system. It consists of the detector (1); fast pre-amplifiers (2) matched to the delay line; low-noise amplifiers (3); fast amplitude discriminators (4) which define a lower limit for the signal, higher than the electronic noise; and a time-to-voltage converter (6) enabling the information to be passed on to a multichannel analyzer or computer (7).

Examples of recording from the one-dimensional counter are shown in Plates 15A,15B and 16.

Circular detector

Plate 17 shows the classical experimental system for recording scattering patterns with circular symmetry. In place of this we use a two-dimensional detector in which the cathode strips are in the form of concentric circles, and the anode system in front is a series of parallel wires (Plate 18). In addition to the advantages described above, the detector enables circular integration of the scattered radiation to be obtained automatically and recorded at very high speed.

Two-dimensional detector

This represents our most recent development and is being used at the DESY outstation for evaluation of the method though its performance is at present limited by the amount of fast computer memory available there. It is illustrated in Plate 19; two perpendicular cathode wire planes, one on each side of the anode wire plane, locate the two co-ordinates of the ionizing events, each using an electronic system similar to that employed with the linear detector. The final picture is built up in the computer. Plate 20 is the first two-dimensional picture obtained at DESY with this detector. (It is intended to use a similar system for detecting neutrons at the ILL.)

Future plans

Future plans include the development of micro-channel plates for recording x-ray patterns and electron microscope images, and of semiconductor detectors which resolve a pattern of energy distribution instead of angular distribution.

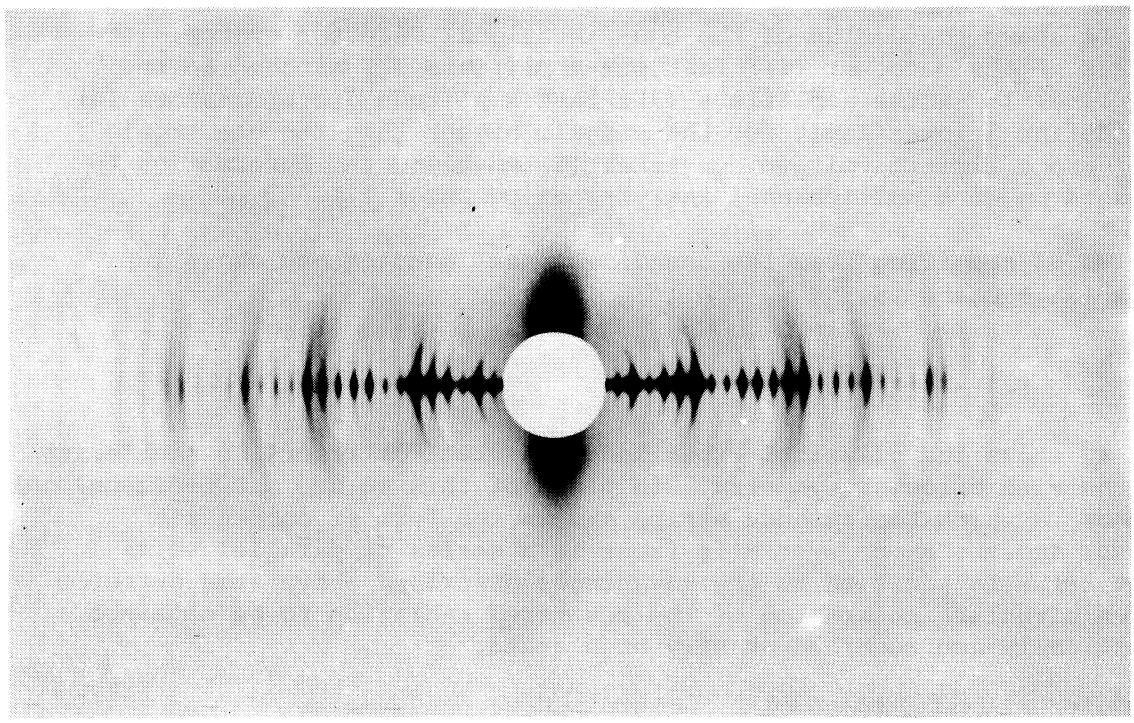


Plate 15A

Meridional reflexions of rat-tail collagen (stained with phosphotungstic acid), recorded on photographic film. The three innermost reflexions are hidden by the beam stop, the first visible reflexion being the fourth order (by courtesy of A Miller).

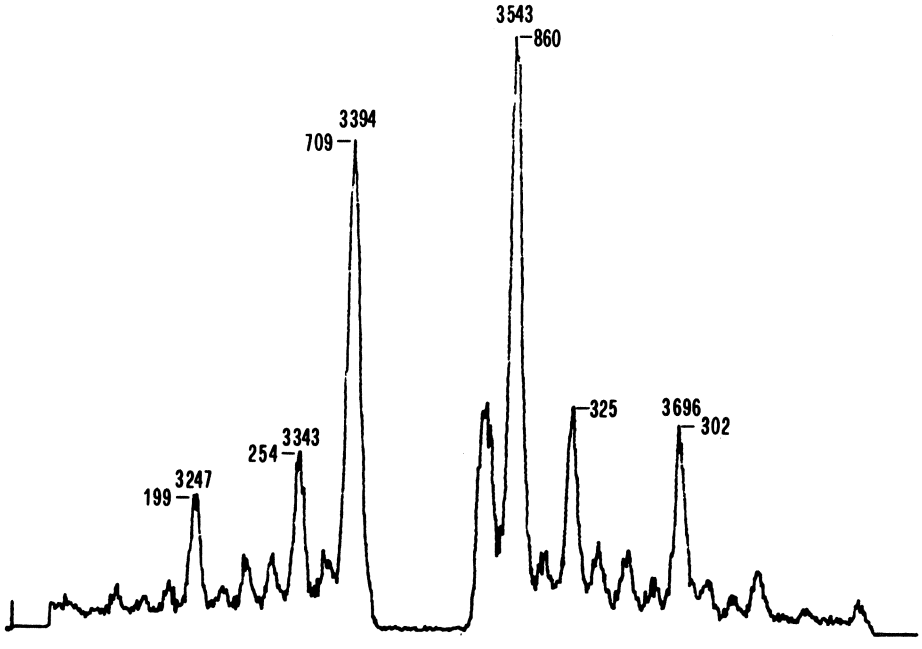


Plate 15B

Meridional reflexion of collagen recorded with a position-sensitive counter filled with argon (the exposure time of 300 sec would be reduced by a factor of 5 with xenon filling). The inmost reflexion on the left hand side is the third order (by courtesy of A Miller).

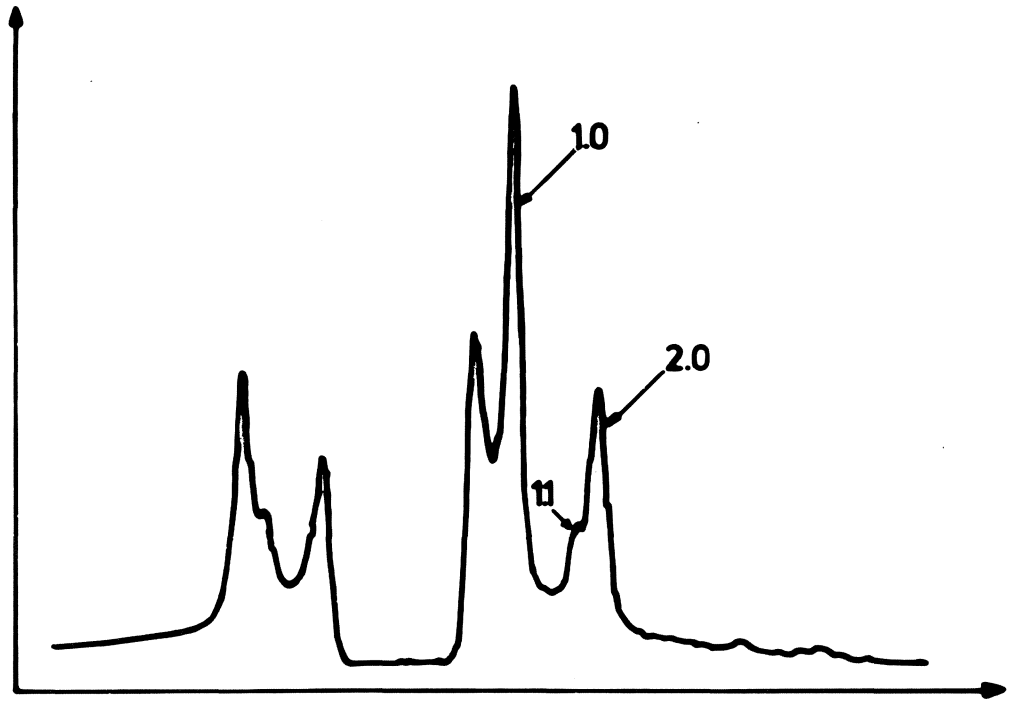


Plate 16

Low-angle diffraction pattern of an insect flight muscle in rigor; three reflexions of the hexagonal lattice are indexed. DESY synchrotron, specimen/detector distance 80 cm (by courtesy of R Goody).

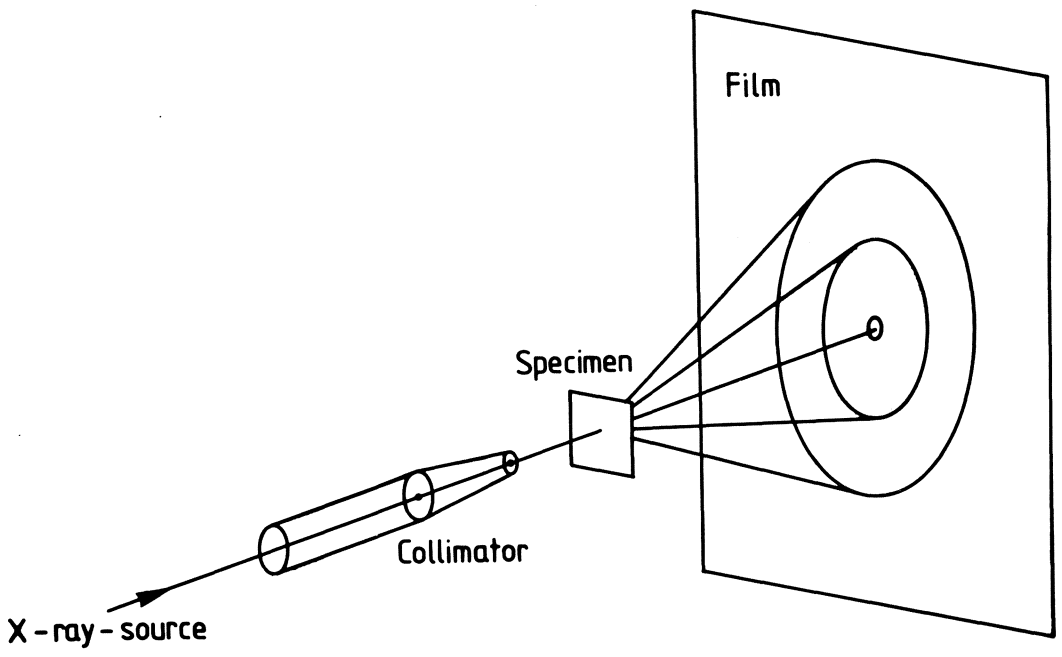


Plate 17

Experimental set-up for photographic recording of centrosymmetrical s ttering patterns.

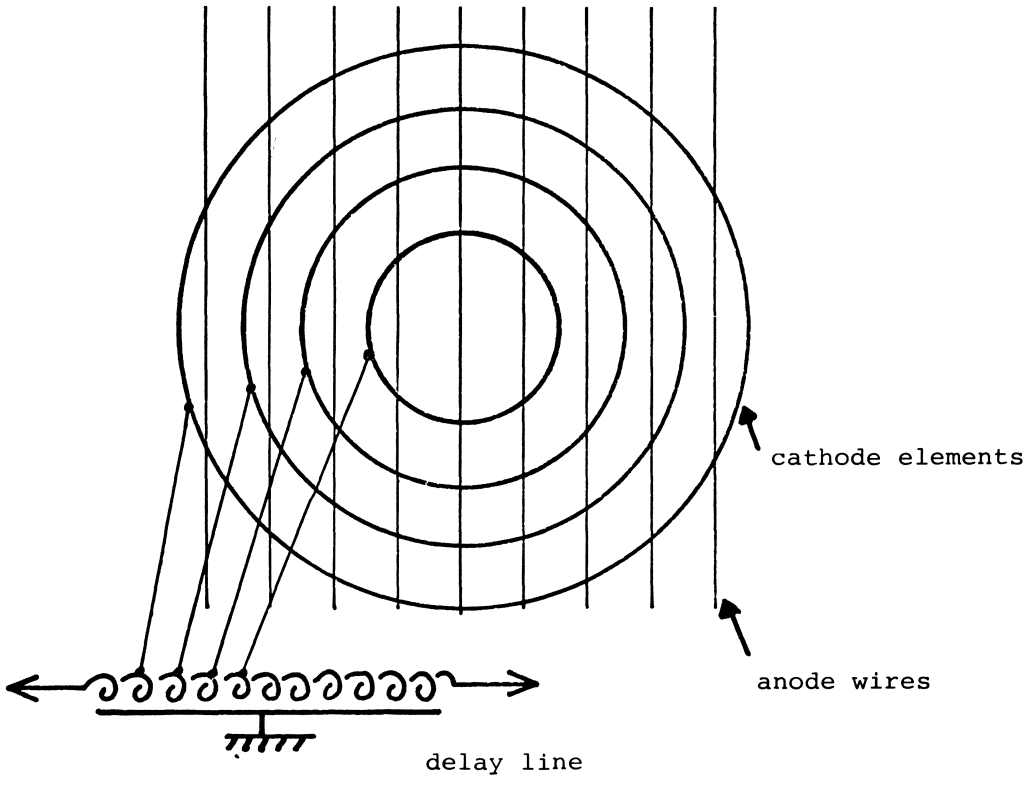


Plate 18

Sketch of circular detector

2 DIMENSIONAL DETECTOR SCHEMATIC

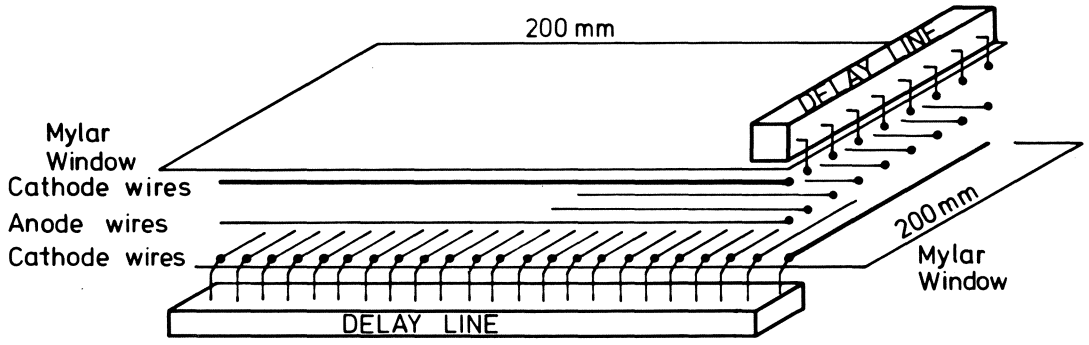


Plate 19

Sketch of two-dimensional detector

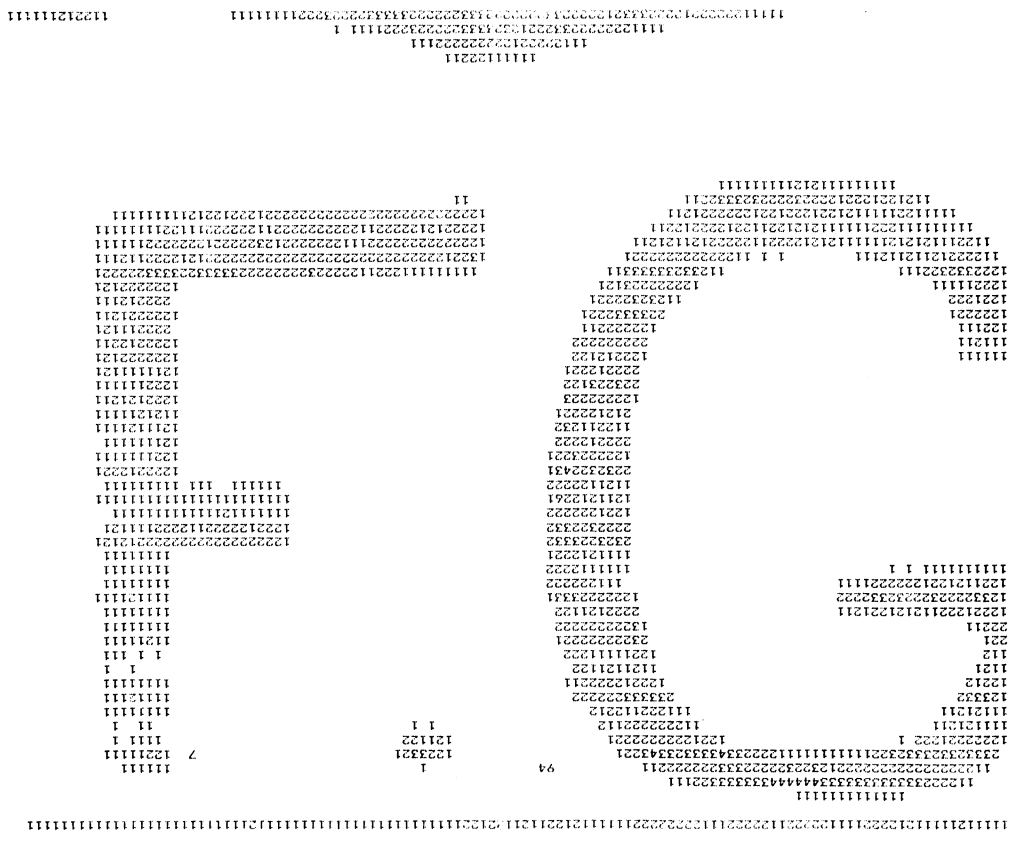


Plate 20

Image of the letters 'FG', recorded with the two-dimensional detector

Computer group

Members: R Herzog*, C Boulin*, F Herzog* (part-time), R Ladner*, P Taylor*

This group was established in February 1976 and most of its activity during the year consisted in selecting and purchasing hardware, recruiting staff, and deciding computer policies. After a comprehensive survey of the European and American minicomputer markets, two NORD 10 computers (manufactured by NORSK DATA A/S) were purchased, following the example of CERN and DESY. The computers were installed before the end of the year, and basic software was prepared.

Three projects were begun:

1 Interfacing the STEM to the NORD 10 (see p 41).

The aim is on-line picture acquisition and display; with the STEM digital image storage is necessary to reduce sample degradation by the electron beam. The project is still in the design stage, but hardware construction is due to start in the second quarter of 1977. To acquire useful images in a reasonable time, on average 2048 x 2048 picture elements in 10 sec, it will be necessary to extend the present limits of technology. Provision will be made for simultaneous recording from more than one signal source (e.g. transmitted, secondary and x-ray), so as to provide correlated information that can be analyzed quantitatively. The techniques to be used will draw on experience of picture processing techniques developed for the analysis of images transmitted from space probes.

2 Interfacing drum microdensitometers for x-ray and transmission E/M photographs

This interfacing, which is also required by the two Outstations, makes use of the CAMAC system. The software, which will be adapted from already existing packages, will also be useful for STEM images; its implementation will present some difficulties owing to the large image sizes involved (2048 x 2048 picture elements or more for low-dose transmission electron microscopy). The basic operations that will be made available to the user are grey-scale display on screen and paper, histogram plotting, contrast correction, 1- and 2-dimensional Fourier transformation, etc. The hardware for the interface has been completed, and the preparation of the data acquisition software has been begun with the help of the EP Division at CERN.

3 Molecular modelling

The aim is to make it possible to construct, view and manipulate models of molecules, in particular biological macromolecules, on a computer display

in relation to the corresponding electron density maps. The project is at present being carried out with an Evans & Sutherland Picture System, supported by a PDP-11/40 computer, at the Deutsches Krebsforschungszentrum in Heidelberg. The system provides hardware-supported real-time rotation and, when necessary, stereograms or orthogonal views. A "package" of programs has kindly been provided by Dr R Diamond of the MRC Laboratory at Cambridge and is under modification to suit the research project for which the system is initially being developed. This is the trial-and-error fitting of a model of the filamentous bacterial virus Pf1 to the x-ray diffraction data, and if possible discrimination between alternative models of the structure (see "Structure and assembly of filamentous bacterial viruses", p 33). Modification of the programs to suit a low-resolution model are required, and also to provide the facility to view several copies of the virus related by symmetry elements so that the intermolecular packing can be studied. The system will be required in the future for other projects, involving both high- and low-resolution electron density maps.

THE OUTSTATIONS

The Outstation at DESY, Hamburg

Head: K C Holmes (until 31 September) (part-time)
H Stuhrmann (from 1 October)

Scientific staff: A Harmsen, J Hendrix*, J Rosenbaum

Technical assistants: W Behrens, P Bendall, R Chors*, E Dorrington*, H Ludwig*, V Renkwitz, B Robrahn

Postdoctoral fellow: Z Rek

Visiting workers: J Barrington Leigh (Heidelberg), R Bowitz (Heidelberg), R Goody (Heidelberg), R Guariguata (Heidelberg), W Hofmann (Heidelberg), J Lowy (Aarhus), H-G Mannherz (Heidelberg), F Poulsen (Aarhus), H Riedl (Heidelberg), R Tregear (Oxford)

In accordance with the basic philosophy of the European Molecular Biology Laboratory to engage particularly in activities that are difficult or impossible to pursue in national laboratories, the EMBL Outstation at the Deutsches Elektronensynchrotron (DESY) is exploiting synchrotron radiation for biological research. Synchrotron radiation indeed has outstanding properties that make it a unique tool for research into the structure and dynamics of biological systems. These special properties were reviewed in the 1975 Annual Report (page 26) and they can be summarized as follows:

1 continuous spectrum from the infra-red region into the region of hard x-rays;

2 strong collimation in the instantaneous direction of flight (typically 1 mrad);

3 linear polarization in the plane of the orbit, and elliptical polarization above and below the plane of the orbit

4 pronounced time structure which is a copy of the pulse structure of the electron beam (pulses as short as 100 psec).

The EMBL Outstation uses that part of the spectrum which passes 2 x 300 μ beryllium windows. The range of wavelengths extends from 3 to 0.01 Å, and is comparable to that of ordinary x-ray sources. In this high energy part of the spectrum the vertical collimation angle is as small as 0.1 milliradian, so that the beam height is only a few millimetres at a distance of 30 m from the source.

The number of photons in an electron volt interval crossing the beryllium window is much higher than with x-ray tubes and attains some 10^{10} or 10^{12} photons/(second x electron volt x milliradian x milliradian) under normal conditions with the synchrotron DESY or the storage ring DORIS respectively

(Plate 21). The total number of photons passing the beryllium window is about 2000 to 5000 times higher, yielding values of some 10^{14} or 10^{16} photons/(second x milliradian x milliradian) respectively.

Even without focussing optics, very high intensities of the "white beam" are available, especially at the storage ring DORIS; at a distance of 30 m from the source, 10^{12} photons/second would pass an area of 0.3 mm x 0.3 mm. The full half-width of the spectrum (dotted line in Plate 21) is rather broad.

There are two steps of monochromatization. Total reflexion by a quartz mirror at extremely small angles (about 10 minutes of arc) will discriminate against the high energy of the x-ray spectrum. The full half-width of the spectrum $\Delta E/E$ will decrease to about 0.4 if convenient filters are used in addition. As biological samples often consist mostly of water, the low energy x-rays are preferentially absorbed. In most cases the sample thickness must be chosen in such a way that it acts as an optimal filter (Plate 22). A more efficient monochromatization is achieved by germanium crystal monochromators, with which the energy resolution $\Delta E/E$ is about 0.1%. The intensity impinging on a typical specimen and focussed to 300 x 200 μ^2 is about 2.10^9 photons/sec under good machine conditions, using the synchrotron as a source.

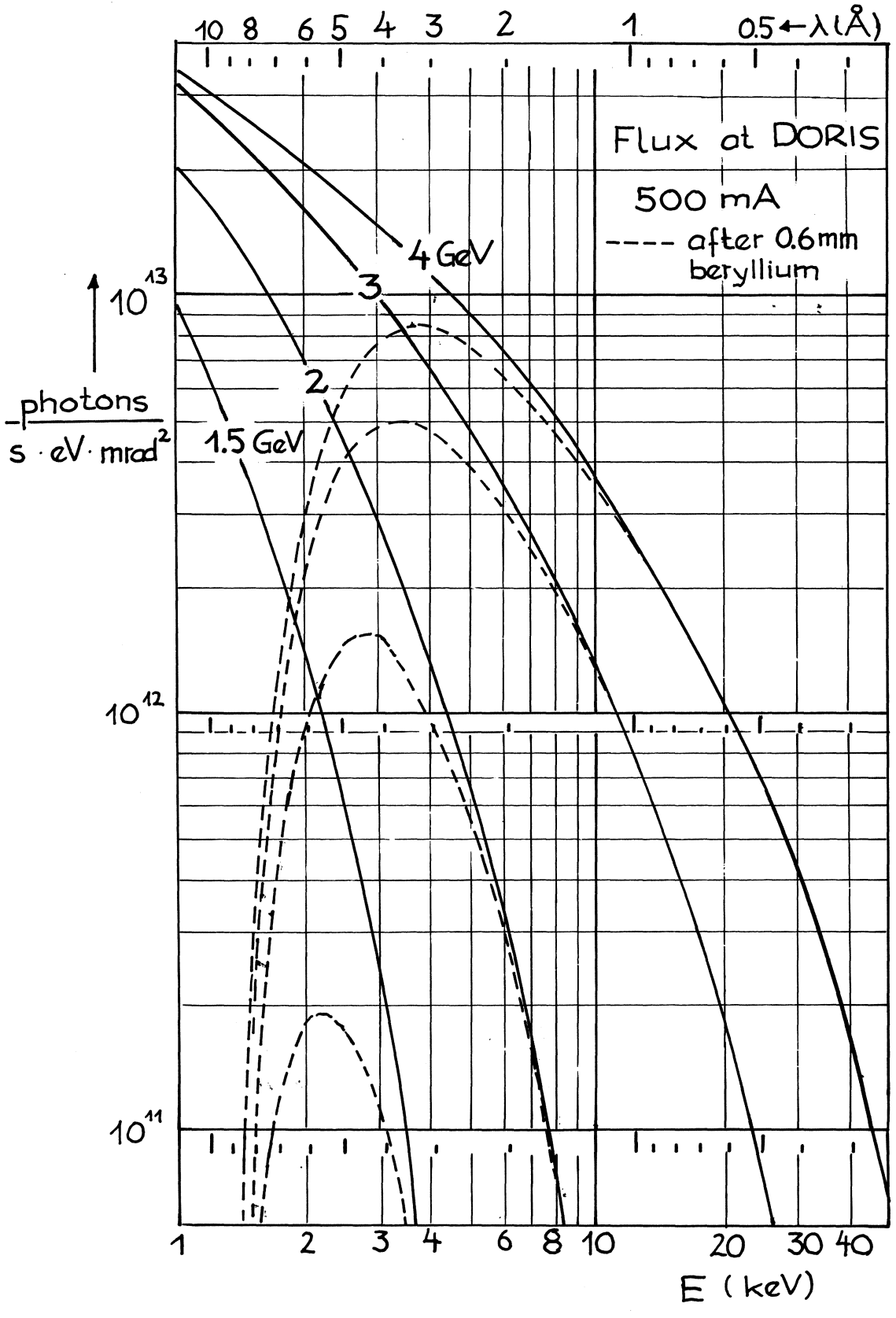
Until now the main emphasis of the research with synchrotron radiation has been on diffraction experiments, especially with muscle[1] and other fibrous systems[2,3] for which the technique has been particularly useful (see below). In addition a study has been made of the value of synchrotron radiation for crystal structure analysis[4], the general conclusion being that investigation of structures with large unit cells (more than 100 Å) will profit from the high intensity of well-collimated and monochromatized synchrotron radiation.

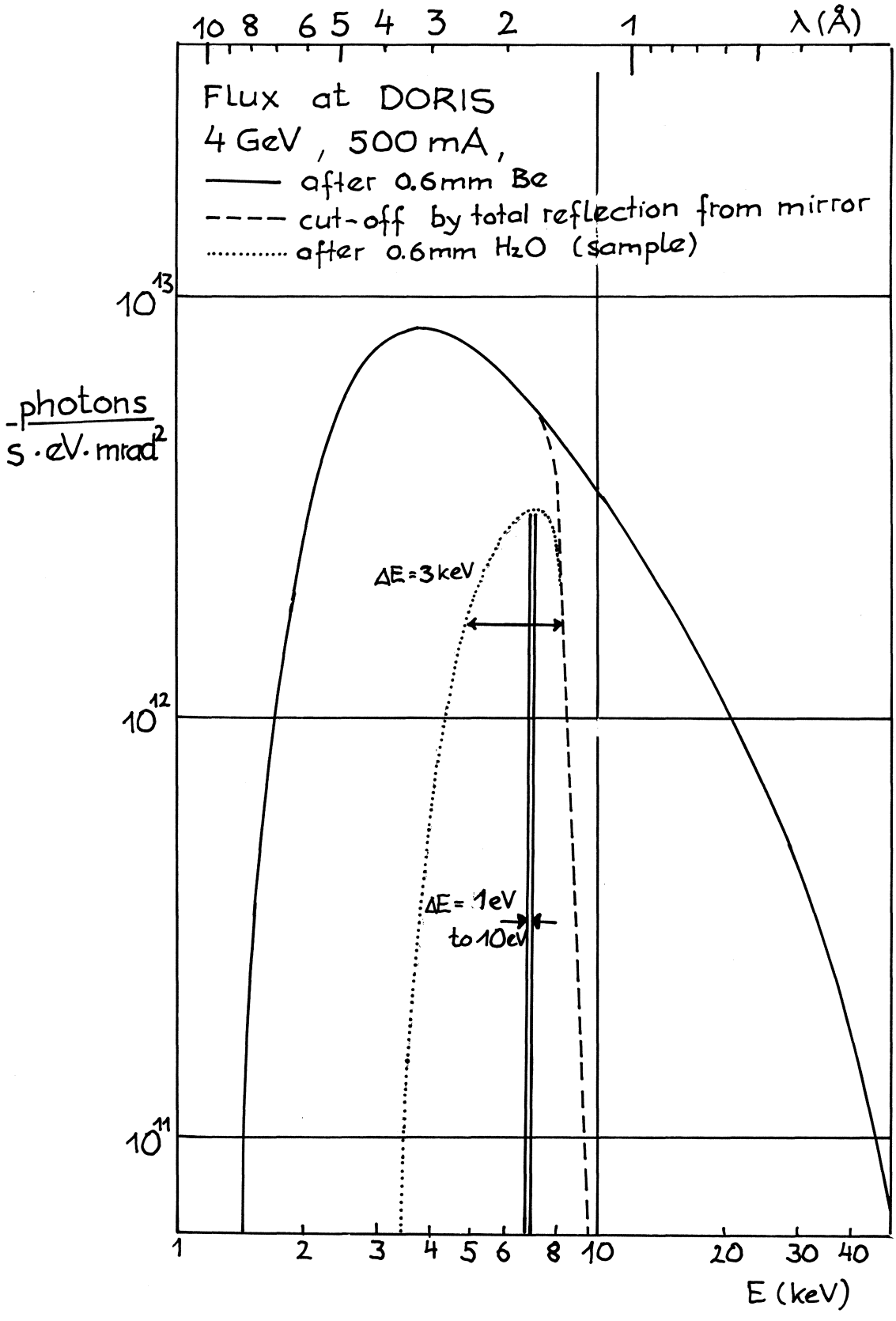
In contrast, synchrotron radiation has not yet been exploited for scattering studies. An energy width of about 10% would be ideal for most diffuse small-angle scattering experiments. The gain in intensity of more than two orders of magnitude compared to crystal diffraction should make this technique a useful tool for the study of relaxation processes by stopped-flow techniques, pressure and temperature jumps, photolysis etc.

Finally, there is the possibility of a renaissance of spectroscopic methods which can take advantage of the continuous spectrum of synchrotron radiation. Thus the analysis of x-ray absorption fine structure is being developed in several laboratories.

Instrumentation development

In addition to the existing small-angle diffraction optical bench (Mark I) in bunker 2 (at the synchrotron), a new optical bench (Mark II) is being installed in bunker 4 (at the storage ring). The planning and construction of the new optical bench have been carried out by G Rosenbaum and the mechanical workshop at EMBL((Heidelberg). It incorporates important improvements, including the possibility of free choice of wavelengths


10 8 6 5 4 3 2 1 0.5 ← λ (Å)
Flux at DORIS
500 mA
---- after 0.6mm beryllium
4 GeV
3
2
1.5 GeV
10^13
photons / s · eV · mrad^2
10^12
10^11
1 2 3 4 6 8 10 20 30 40
E (keV)


10 8 6 5 4 3 2 1 λ(Å)
Flux at DORIS
4 GeV , 500 mA ,
after 0.6mm Be
cut-off by total reflection from mirror
after 0.6mm H2O (sample)
10^13
10^12
10^11
photons / s · eV · mrad^2
ΔE = 3 keV
ΔE = 1 eV to 10 eV
1 2 3 4 5 6 8 10 20 30 40
E (keV)

from 0.5 to 2.2 Å and of automatic collimation control. In the summer of 1977, the prototype in bunker 2 will be replaced by a new optical bench for small-angle diffraction (Plate 23). Both bunkers will have small-angle cameras by April 1977. A special camera for muscle diffraction, belonging to H E Huxley, has been moved to the Outstation from NINA (Daresbury, UK) and will be set up at the storage ring in March 1977 (Plate 24). An optical bench for use with an oscillation camera has been built in the EMBL workshop at Heidelberg and will soon be installed in bunker 2.

It will be possible to carry out EXAFS experiments (extended x-ray absorption fine structure) in both bunkers by late summer 1977.

Special projects using the time structure of synchrotron radiation in the nanosecond region are in progress. The investigation of delayed diffraction patterns from certain nuclei, e.g. ^{57}Fe, appears to be most interesting. Promising applications can also be expected for photolysis in general.

Important progress has been achieved in the development of two-dimensional position-sensitive counters. Initial tests of the delay-line detector built by A Gabriel gave excellent results in linearity, though the degree of homogeneity still leaves room for improvement. Data are collected by an IN90 computer (Intertechnique) and are written on magnetic tape. At present one image contains 8192 elements but, after modification of the IN90 system, 16394 elements will be available. Two-dimensional display of the scattering pattern is carried out on a PDP11/45.

Faster data acquisition is possible with a ring detector. The ring geometry of this detector is appropriate for isotropic small-angle scattering, e.g. of solutions. Parts of the detector system have been built and tested by J Hendrix and A Gabriel, and it should be operational by summer 1977.

A position-sensitive counter with higher resolution is desirable for diffraction patterns containing much detail. A CAMAC data acquisition system has been planned by J Hendrix and a detector system with 256 x 256 picture elements will be built in close collaboration with DESY, ILL (Grenoble), CERN (Geneva) and the Hahn-Meitner-Institut für Kernforschung (Berlin). This detector system should be operational by the end of 1977.

Plans for the extension of the range of synchrotron beam facilities were outlined in a letter to EMBO members in December 1976.

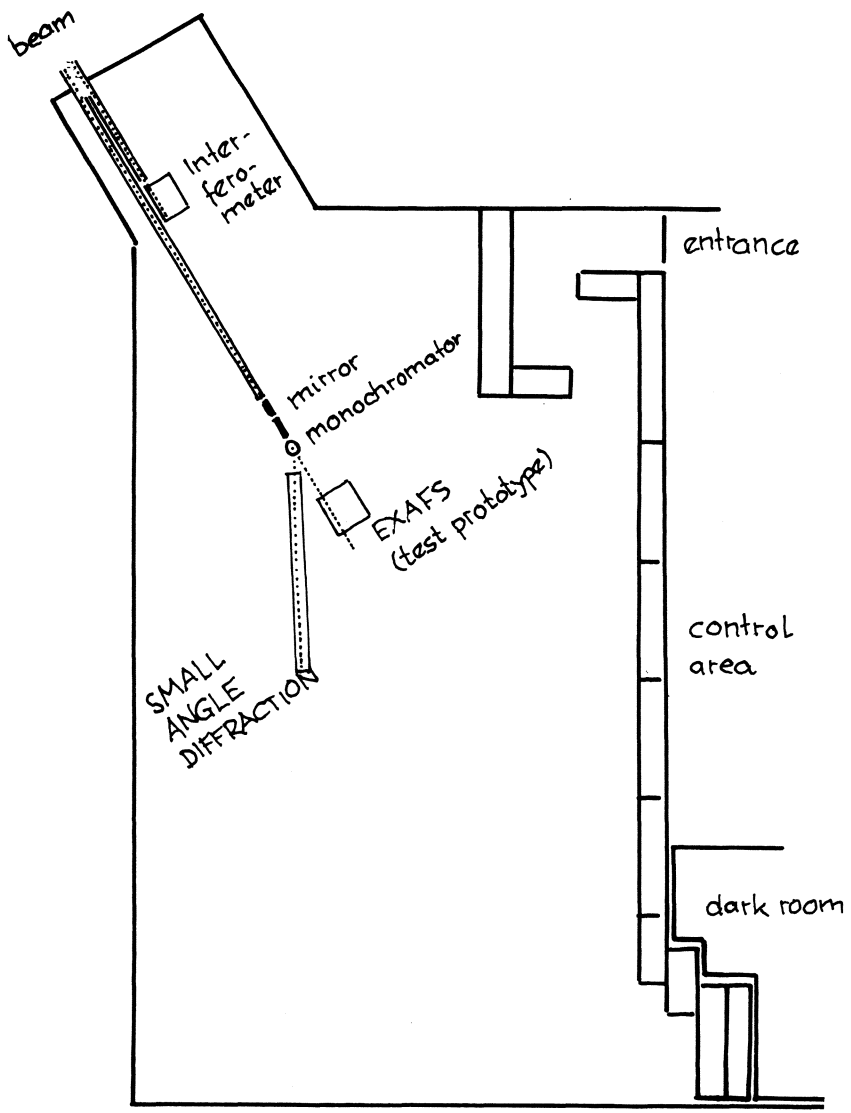
Group reports

1 Optical benches and control circuits

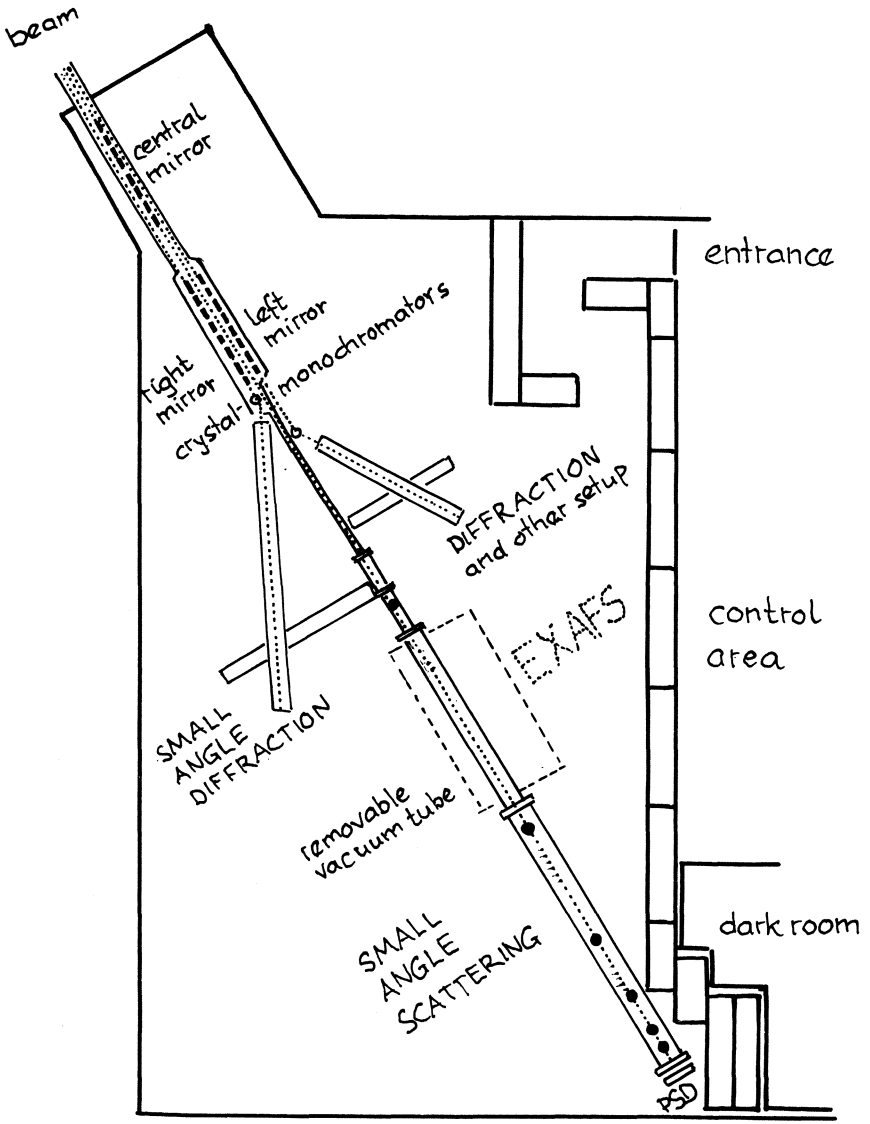
G Rosenbaum, J Hendrix, R Chors*

The technological program outlined in the 1975 report was continued. The remote control circuits of the Mark I optical bench in bunker 2 (synchrotron)

BUNKER 2 at the synchrotron DESY



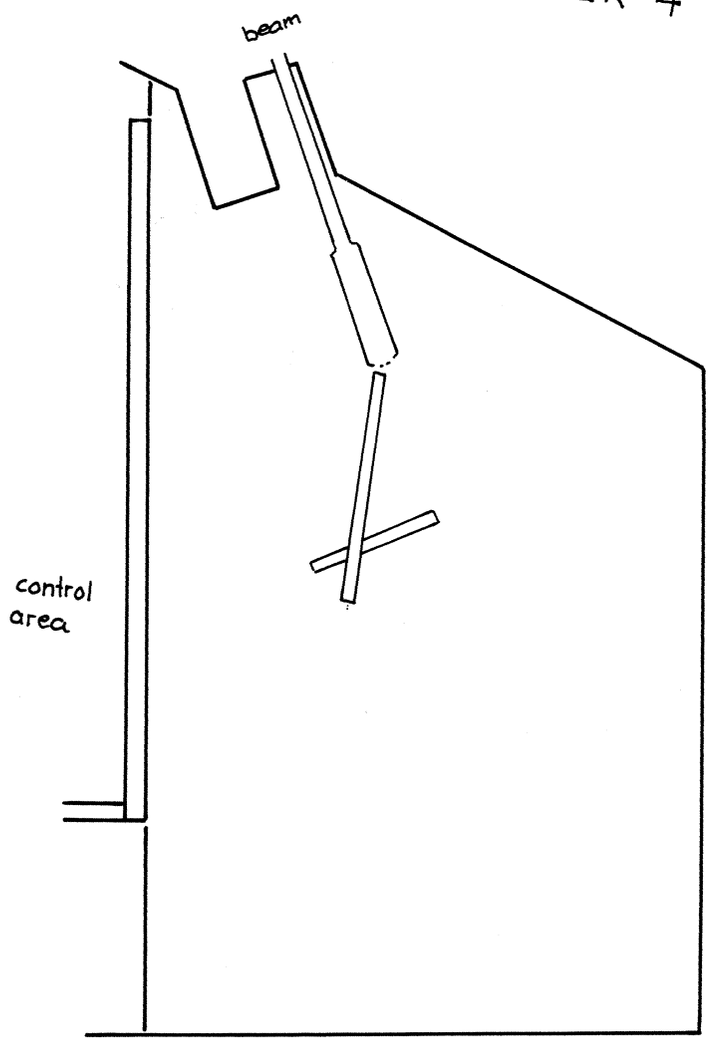
23A Facilities available in 1976



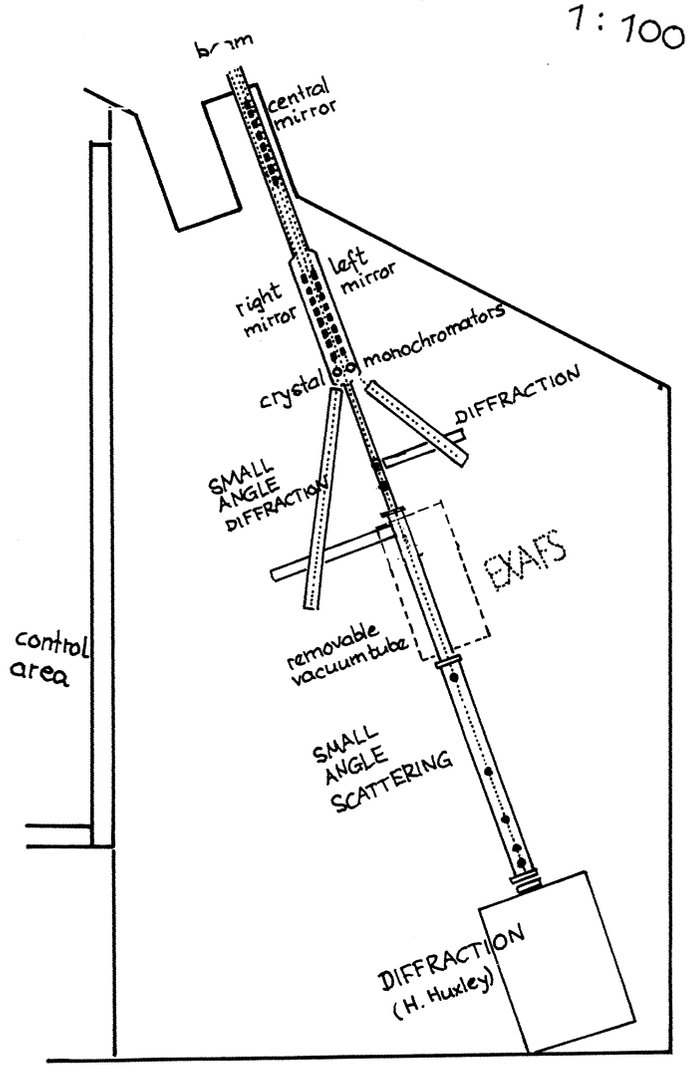
23B Facilities under construction

BUNKER 4 at the storage ring DORIS

1 : 100



24A Facilities available in 1976



24B Facilities under construction

were completely overhauled. The control circuits (including computer control) for the Mark II optical bench were designed and built (in collaboration with C Stettner of the EMBL electronic workshop at Heidelberg, and P Bendall). The Mark II optical bench was delivered to Hamburg from Heidelberg in July to be installed in bunker 4 (storage ring); the control circuits were delivered in November and commissioning of the whole system is in progress.

2 X-ray monochromators and x-ray flux measurements

A Harmsen, Z Rek

A theoretical study of the parameters influencing monochromator band-width was undertaken with a view to constructing monochromators with a relatively wider band pass than is available from quartz.

Channel-cut crystals for EXAFS have been tested on a specially built remotely controlled goniometer suitable for measuring rocking curves. This has been used in conjunction with an energy discriminating solid state detector which is of great help in controlling the effects of unwanted x-ray fluorescence.

A germanium 111 crystal has been installed in the Mark I optical bench in place of the quartz monochromator. This has trebled the beam intensity available from the synchrotron by increasing the bandwidth.

A series of transparent ionisation chambers has been developed for beam monitoring. These are invaluable for positioning slits and specimen. They have not yet been calibrated absolutely but the indication is that, under the best conditions, a flux of 2×10^9 photons of monochromatic focussed radiation is available from the synchrotron on the Mark I optical bench.

3 Insect flight muscle group

R Goody, J Barrington Leigh, H G Mannherz, G Rosenbaum, R Tregear

Using the one-dimensional position-sensitive counter of Gabriel and the Mark I optical bench, and observing the ratio of the strong equatorial $10\bar{1}0$ and $20\bar{2}0$ reflexions, it has been possible to titrate the binding of the ATP analogue ATP (β,γ)-NH to insect muscle fibres[5]. This has been done over a range of temperatures, making it possible to calculate the enthalpy of binding, an important parameter for indicating the close similarity between this analogue and ATP in their binding to the active site.

Making use of the great intensity of the x-ray beam and the very high angular resolution of the Mark I optical bench, it has been possible to obtain very detailed x-ray diagrams of the fibres in the relaxed state (see Plate 25). These new observations, coupled with a theoretical study

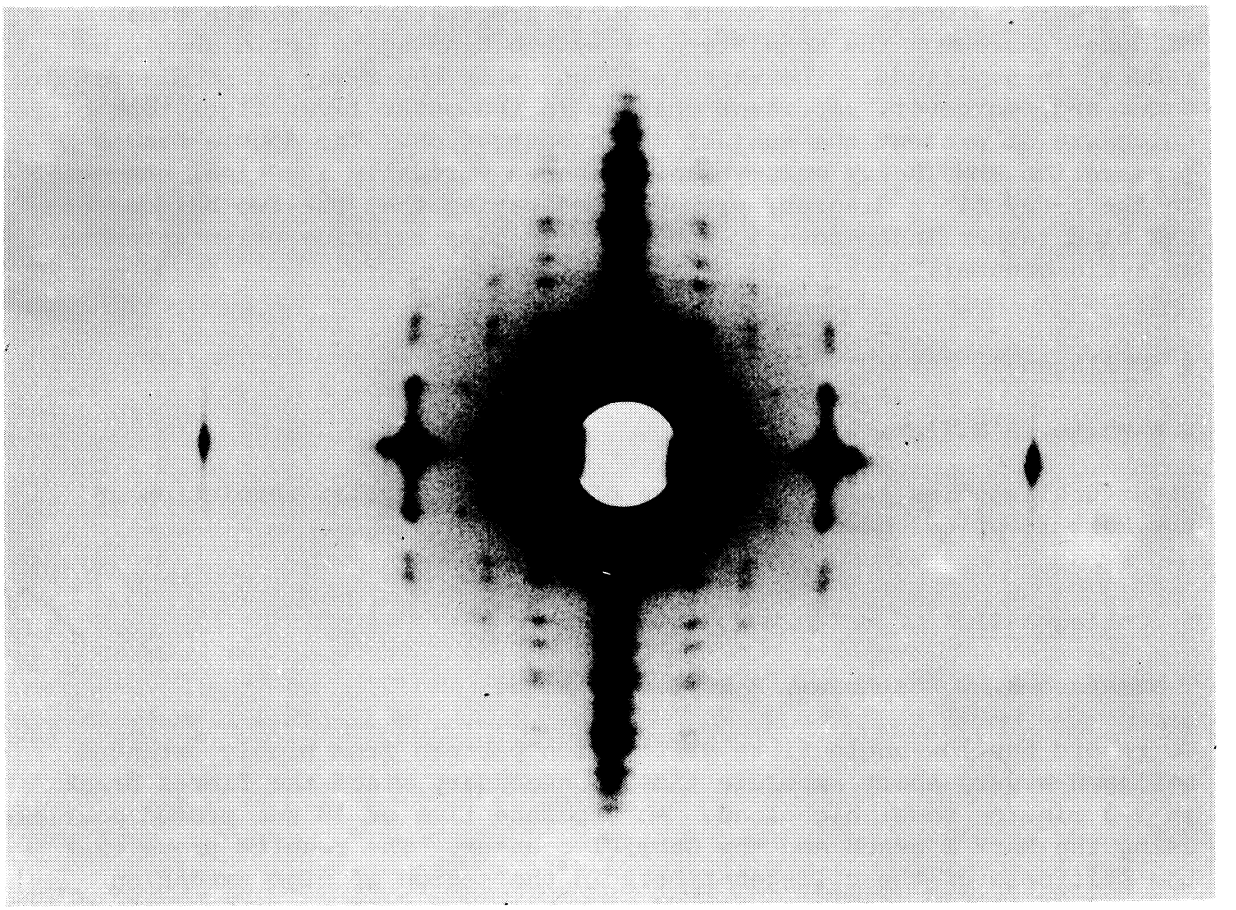


Plate 25

Low angle x-ray diffraction pattern from glycerinated insect flight muscle in the relaxed state, taken at the EMBL Laboratory at DESY, Hamburg. Conditions: 15 mM ATP, pH 6.9, 4°C. Synchrotron conditions: 7.2 GeV and 8 mA, exposure time 2 h.

of the diffraction pattern, indicate that a reappraisal of the evidence for the symmetry of the myosin helix is necessary. They also show that the degree of interaction of actin and myosin in the relaxed state is much greater than had been supposed.

A basis of the theoretical reappraisal of the x-ray fibre diagram from insect muscle fibres was to suppose that the actomyosin interaction in rigor was modulated along the length of the filament. To check this, fibres were saturated with S1 (a soluble preparation of myosin cross bridges) whereupon the modulation of myosin binding to actin should be reduced in amplitude. The expected changes in the x-ray fibre diagram did occur and, moreover, important changes in the outer layer lines took place which are at present the subject of investigation. The double-headed preparation HMM (heavy meromyosin) produced virtually identical changes in the x-ray fibre diagram, probably indicating that the two bridges of HMM bind rather independently and with the same relative orientation to an actin monomer.

4 Collagen I

S W White, A Miller, A Harmsen

Structural studies taking advantage of the high angular resolution of the Mark I optical bench are continuing.

5 Collagen II

T Nemetschek, G Rosenbaum, R Bowitz, H Riedl

To record the low-angle fibre diffraction pattern from highly extended collagen a very short exposure time is necessary since the fibres break in 2-3 minutes under high load. An exposure time of 10 sec proved possible using the Mark I bench and the Gabriel counter. The results prove that the stiffness of the collagen fibres in the region of high extension (more than 3%) is directly related to the extensibility of the collagen triple helix.

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5 Goody, R. S., Barrington Leigh, J., Mannherz, H-G., Tregear, R. T. and Rosenbaum, G. (1976). *Nature (London)*, 262, 613-615

The Outstation at ILL, Grenoble

Head: A Miller

Scientific staff: C Berthet, H Lindley* (part-time), H Ngotri*, D Tocchetti

Technical assistants: H Krischke*, J Sedita

Postdoctoral fellows: D Hulmes, S Cusack

Postgraduate student: S White

Student: P Blanc

Visiting workers: B Doyle (NIH, USA), K A Piez (NIH, USA), A Veis, (Northwestern University, USA)

In October 1976 it was announced to ILL neutron users that the EMBL Outstation was ready for use; its equipment had been installed and was in working order. By the end of the year there were working visits from seven different groups of ILL users, all external to EMBL. These users came from laboratories in France, UK, Belgium, Italy and the USSR (see below). The only major items of equipment still outstanding are the computer and the image analysis system.

The research program of the Outstation itself is directed towards the study of biological fibres, principally connective tissue and muscle. Connective tissue is composed mainly of collagen. The characteristic collagen fibrils, as seen in the electron microscope, are indefinitely long and about 1500 Å in diameter, and exhibit a banding of period 670 Å. The collagen molecules themselves are 3000 Å long and 13 Å in diameter. It is now possible to understand how the amino-acid sequence of the collagen molecule dictates its self-assembly into the long fibrils of connective tissue. Furthermore, it can be seen how the amino-acid profile can be modified by the medium or by the presence of other macromolecules so that the collagen molecules may be driven to aggregate in one or other of a variety of polymorphic forms[1]. We have determined by low-angle x-ray analysis the structure of the fibrils in one dimension (i.e. projected on to the fibril axis) to virtually amino-acid resolution[2]. This has provided information about the conformation of the non-helical regions of the molecule in the native fibrils, and the structural model is being tested and extended by a low-angle neutron diffraction study[3]. We are now involved[4] in using this knowledge to examine how and where mineralization occurs in connective tissue during the development of bone or other hard tissues. In addition we are using a combination of x-ray diffraction (C Berthet) and electron microscopy (D J S Hulmes) to try to determine the complete three-dimensional packing of the collagen molecule in the fibrils.

Three other novel types of experiment have been started. In the first, the very low-angle neutron camera at ILL (specimen to detector distance 40 m) has been used to record diffraction maxima from collagen corresponding to spacings of 5000 Å. This pattern is due to the lateral arrangement of the fibrils, and of course the intensity maximum corresponding to 5000 Å overlaps with the region of reciprocal space observable with visible light. This establishes in practice an overlap between neutron and light diffraction and we intend (in collaboration with H Stuhrmann, of EMBL, Hamburg) to establish an overlap also with x-ray scattering from collagen. The present results are being used to study the density of collagen fibrils, which is relevant to the problem of the three-dimensional molecular packing referred to above.

In the remaining two sets of experiments we are enjoying the collaboration of J W White (ILL) and his co-workers. These experiments involve measurements of inelastically scattered neutrons and light, and reveal that these two methods may also overlap in the range of energy transfer which they explore. Measurements of inelastically scattered neutrons have been made at ILL by instruments with energy resolution of 60 μeV and 1 μeV respectively. Quasi-elastic broadening of the incident wavelength was readily detected from tendons at various humidities, and noted to be of two types, one of the order 100-200 μeV and the other of the order 1 μeV. The first can be ascribed to the movement of water molecules in the tendon and the second to segmental motion of the collagen molecules. The Q-dependence of these effects is being investigated. Brillouin light-scattering from collagen has been measured (by D James (Queensland, Australia) and R Harley (Oxford)) using a triple pass Fabry-Perot interferometer. Intensity maxima are observable with energy displaced by 0.29 - 0.33 cm^{-1} (28 - 40 μeV) from the energy of the incident light (λ = 5145 Å). These excitations are probably due to longitudinal acoustic phonons propagating along the fibrils, and they lead to an estimate of 9 x 10^{10} dynes cm^{-2} for the microscopic elastic modulus of collagen. The aim of this work is to relate the microscopic structure and dynamics of collagen to the mechanical properties of connective tissue.

In the biochemical laboratory, H Lindley has started a project aimed at the determination of the amino-acid sequence of the rod portion of the myosin molecule. The object is to permit the kind of analysis that was successful in collagen and tropomyosin, in order to clarify the nature of the intermolecular interactions. At present this project is in its early stages and novel methods for obtaining suitable peptides are being explored.

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Users of the Outstation facilities:

S Bram (Paris)
B Jacrot (Grenoble)
M Parfait (Louvain, Belgium)
C Rodger (Oxford)
N Roveri (Bologna)
I Serdyuk (Moscow)
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