INSTITUT DE LA VIE

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"DE LA PHYSIQUE THEORIQUE A LA BIOLOGIE"

VERSAILLES : 4-8 JUILLET 1988

RESUMES DES RAPPORTS

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HIGH TEMPERATURE SUPERCONDUCTIVITY: CURRENT THEORETICAL SITUATION

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Abstract

The discovery of superconductivity in ceramic oxides has led to significantly higher superconducting transition temperatures. Dozens of theoretical proposals have been made, but at present, there is no consensus on the underlying mechanism causing the high transition temperatures. After a brief review of the properties of superconductors and the microcopic BCS theory of superconductivity, the present status of the theory of high temperature superconductivity will be discussed.

Introduction

The rapid recognition and large response to the discovery [1] of superconductivity in ceramic oxides in 1986 are exemplified by the award of the 1987 Nobel Prize in Physics to Bednorz and Mueller and the large number of new research papers written in this area. The original observation [1] of superconductivity in La-Ba-Cu-O around 35K was followed by a breaking of the "nitrogen barrier" with the discovery [2] of superconductivity in Y-Ba-Cu-O above the boiling point of nitrogen in the 95K range. Now the superconducting transition temperature, T_c, has been raised [3] above 120K. A number of higher temperature observations of superconductivity have been reported, but at present, these are not reproducible.

On the experimental side, the properties of both the normal and superconducting phases of the oxides have been studied extensively [4,5]. Theoretical explanations for the higher T_c's have been suggested [4], but at this point, there is no general agreement on a theoretical explanation of the phenomena. This is contrasted with the situation before 1986 when it was felt that the BCS theory of superconductivity [6] explained almost all the observations in this field, and it became possible recently to predict the existence of new superconductors [7].

There is considerable motivation in this area to achieve room temperature superconductivity in the future and to develop applications now with liquid nitrogen replacing liquid helium as a coolant. These technological considerations have added to the perceived importance of this field. From a more basic scientific point of view, it is difficult to assess the impact of this discovery. The BCS theory [6] and other concepts related to the microscopic understanding of superconductivity gave great insight into the properties of matter ranging from elementary particles to nuclei to atoms, molecules, and solids. If the new superconductors are shown to be similar to their predecessors, except for some simple scale changes, the discovery

would still be important but not scientifically "earth shaking." Investigations at this point suggest that there are significant differences between the new and the "old" superconductors. Scaling takes us to the very limits of the domains of the BCS theory, and there is belief by some that radical changes and new ideas are needed. The possible influence of the correct theory explaining the new phenomena depends on the form and content of the theory, but even if it is unexpectedly dull, the search for it so far has raised so many interesting and challenging questions that at the least we are achieving greater understanding of interactions in solids and a deeper knowledge about materials.

Some Properties of Superconductors

A superconductor has zero resistance. This is a necessary but not sufficient condition to define superconductivity. For a hypothetically perfect conductor, according to Maxwell's equations, magnetic fields present in the conductor when it loses its resistance will be trapped inside the material. This is contrasted with the expulsion of internal magnetic fields for superconductors discovered first by Meissner. The Meissner effect and zero resistivity are the two phenomena most often used to establish the existence of superconductivity.

Other important macroscopic properties of superconductors include the destruction of superconductivity by magnetic fields (critical fields), persistent currents in superconducting loops, and the trapping of magnetic flux in holes within a superconductor. The observation of trapped flux bears on the microscopic picture of superconductivity because the trapped flux is quantized in units of hc/2e where h, c, and e are Planck's constant, the velocity of light, and the charge on the electron. The factor 2e illustrates the importance of electron pairs. The binding of electron pairs is also responsible for the infrared and thermal properties of superconductors. For example, a superconducting gap Δ can be determined from optical studies for frequencies $\omega \sim 2\Delta/\hbar$. For most superconductors $2\Delta \sim 3.5\ K_B T_c$, where k_B is Boltzmann's constant and the low temperature heat capacity depends exponentially on Δ.

An experiment which was important to uncovering the microscopic mechanism of superconductivity is the isotope effect. It was found that by varying the isotopic mass M, T_c varied as $M^{-\alpha}$ with $\alpha \sim 0.5$ for most superconductors. Since lattice vibrations vary in this same way, the "isotope effect" showed that superconductivity depended on electron-lattice interactions. Experiments on the tunneling of electrons between superconductors and normal metals or other superconductors verified this and the details of the theory of the interactions.

BCS Theory

Bardeen, Cooper, and Schrieffer (BCS) proposed their theory [6] in 1957, and applications of the theory were successful in almost all areas where it was tested. The theory was refined and extended using field-theoretic approaches, but the basic ideas remained the same. In particular, the theory proposes that in a superconductor electrons form pairs through a mutual attraction caused by the lattice. The mates in a pair are far apart in space compared to the separation of the pairs. For example, in superconducting Al it is estimated that between the mates in an average pair, there

a million electrons participating in other pairs.

The attractive pairing interaction is characterized by a dimensionless coupling constant λ caused by the electron-lattice interaction. A measure of the Coulomb repulsion between electrons is given by μ. In the original BCS formulation, if $\lambda > \mu$, then

$$T_c = 1.13\ T_D\ e^{-\frac{1}{\lambda-\mu}} \qquad (1)$$

where T_D is the Debye temperature characterizing the lattice vibrations. Since $T_D \sim M^{-0.5}$, this explains the isotope effect.

Equation (1) is approximate. Since T_c depends very sensitively on λ and μ, these couplings must be determined very accurately, and the equation for T_c must be capable of evaluating the effects of the coupling with great precision. This has been achieved only in the last few years, and some successful predictions have been made.

High T_c

There were many proposals for increasing T_c before 1986 and theories related to the maximum possible T_c resulting from electron-lattice interactions. These theories were revived after the recent discoveries, and new ones have been added. Some experimental properties of the oxides indicated that new theories were necessary [4]. Among these, the observations [4] that the isotope parameter $\alpha \sim 0.02$ for Y-Ba-Cu-O and $\alpha \sim 0.15$ for La-Sr-Cu-O encouraged the view that electron-lattice interactions are not alone in pairing the electrons. In addition, estimates [8] of the large λ's needed for these systems were discouraging for phonon mechanisms.

Since all the important interactions in solids arise from the Coulomb interactions among the electrons and ions, the mechanisms investigated focused on electron pairs resulting from excitations of: plasma-like oscillations of the electron sea; excitations involving electron-hole pairs (excitions); acoustic-like waves in a system having electrons of different angular momentum or band character (demons); magnetic excitations including spin fluctuations and antiferromagnetic correlations in a spin liquid (resonating valence bonds); and others. Many of these proposals [4] have been examined and refined in the current literature, but as yet the experimental data obtained does not rule out these mechanisms. In addition, there are suggestions that the unusual behavior results from the particular nature of the crystal structure and vibrational structure of these oxide systems. Hence, anharmonic lattice vibrations; defect enhancements of coupling; the two-dimensional character of the Cu-O planes; and one-dimensional Cu-O chains have been suggested. Again, experiment has not distinguished among these, but at present, chain theories are thought to be inadequate.

Conclusions

In the end, decisions in science are made by experiment. It is, therefore, essential that the theoretical proposals be evaluated and their predictions clarified. There are possible measurements to narrow the field, and these may help filter the theories or parts of theories which are applicable.

It is hoped that close experimental-theoretical collaborations will yield answers in the near future.

Acknowledgements

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LUNDI 4 JUILLET 1988

14 h 30 - 18 h

DEVELOPPEMENTS NOUVEAUX EN PHYSIQUE NON LINEAIRE

NEW DEVELOPMENT IN NON LINEAR PHYSICS

STABILISATION OF NON-LINEAR EXCITATION IN BIOLOGICAL SYSTEMS

by

H. Fröhlich

In the first meeting of the Institut de la Vie, about twenty years ago it has been shown that owing to the extraordinary dielectric properties of biological materials, the supply of random energy above a certain rate $S > S_o$ may lead to the coherent excitation of an electric vibration in the region of 10^{11}Hz. taking the form of a phase transition analogous to an Einstein-Bose condensation. Essentially this requires existence of certain non-linear interactions. This paper has recently been listed as a "most frequently cited work".

At the same meeting, Prigogine without specifying a particular type of interaction introduced his systems of "Dissipative Structures" showing also that when certain conditions are satisfied, non linear interactions may yield coherent excitations.

Far reaching developments have taken place since then, and a large book entitled Biological Coherence and Response to External Stimuli, ed. H. Fröhlich, Springer Verlag is at present in print.

From the point of view of physics, biological systems have three general characteristics

1. They are rather stable but far from equilibrium.

2. They exhibit a non-trival order.

3. They have extraordinary dielectric properties.

Three kinds of coherent excitation can then arise:

A. Excitation of a single mode of vibration.

B. Excitation of a metastable highly polar ferroelectric state.

C. Excitation of limit cycles or Lotka-Voltera oscillations in complex systems.

Fairly recently the existence of "Deterministic Chaos" in conjunction with bifurcations has also arisen, and experimental confirmation is now available.

To show reproducibility then requires a considerable number of experiments, and quite likely a relevant general concept has not been found yet.

Experimental evidence for the three types of excitation has been available for some time as discussed e.g. in the above mentioned book. It will be noted that solitons belong to B and a minimum energy is required for this type of excitation. Excitation of type A, however, requires an energy flux S to exceed a minimum S_o, $S > S_o$. It has been shown recently that this is available when temperature differences exist, and examples have been given (Phys. Lett. 110A, 480, 1985) where a small temperature difference yields effects where an overall change in temperature does not.

Most remarkable is a Russian finding according to which very sharp frequency dependent stimulations exist in certain humans at positions that are also active to acupuncture. From personal communications I understand that these effects have been confirmed in thousands of cases and should be interpreted in terms of the theory of coherent excitation.

A further aspect of the importance of coherent excitations arises in the theoretical discussion of cancer as first mentioned in the meeting on Biophysical Aspects of Cancer Prague 1987, Charles University. Assume that each cell of an organ, or tissue, can be excited coherently at the same frequency characteristic for the particular organ as first suggest by F. Fröhlich (Cooperative Phenomena, Ed. H. Haken & M. Wagner, Springer Verlag, 1973). When so excited, through long range coherent interaction, a collective normal mode is established; each cell contributes to it and reversly it keeps each cell excited at the appropriate phase and strength. Now preceding cell division, certain changes must take place in that particular cell which thus may lose its resonance with the normal mode. This requires the supply of energy which may not be available. The coherently excited mode thus controls cell division.

The interaction of the cell vibrating with the correct frequency thus has two consequences: It contributes to the magnitude of the amplitude of the excited mode, and, by it, is kept in the correct phase and strength. If now by external circumstances its frequency is changed, then the total amplitude is reduced, but forces on the particular cell try to bring it back to the correct state. If a great number of cells loose their correct frequency, however, then the excited normal mode will collapse. Control of cell division will no longer exist and cancer may arise.

The situation thus resembles an order-disorder transition. The order parameter is the amplitude of the coherently excited mode; cancer is the disordered state.

Recently it has been reported (P. Newmark, Nature 14 May 1987, 327, 101) that normal cells can exert control over those of their number that have been transformed into cancer cells, a characteristic to be expected in an order-disorder transition, as suggested above. It remains to be verified that the relevant interaction arises from long range coherent excitation.

Biophysics, Cell Physiology, Biometeorology, Engineering, Environmental Physiology

Of interest to biophysicists, cell physiologists, biometeorologists, engineers, environmental physiologists.– Level: Research Monograph

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H.F. FRÖHLICH, Liverpool, UK (Ed.)

Biological Coherence and Response to External Stimuli

1988. 97 figs. Approx. 340 pp. Hard cover DM 228,–.
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Contents: Theoretical Physics and Biology.– Theory of Non-Linear Excitations.– Structures, Correlations and Electromagnetic Interactions in Living Matter; Theory and Applications.– Resonant Cellular Effects of Low Intensity Microwaves.– Biological Effects of Low Intensity Microwaves.– Metastable States of Biopolymers.– Photosynthesis.– Emission of Radiation by Active Cells.– Physiological Signalling Across Cell Membranes and Cooperative Influences of Extremely Low Frequency Electromagnetic Fields.– The Interaction of Living Blood Cells.– The Genetic Code as Language.– Electromagnetic Effects in Humans.– Coherent Properties of Energy – Coupling Membrane Systems.– Coherent Excitations in Microtubula; Implications for Biological Information Processing.

This book presents an extensive treatment of the introduction of modern physical concepts into biology. In particular, the concept of coherence finds wide applications and yields novel results in context with multiple problems as they arise in biology: these include long range resonant cellular effects and resonant interactions of biological tissues with low intensity electro-magnetic radiation. Extensive experimental support of the theoretical concept is presented.

Dieses Buch befaßt sich mit der Anwendung moderner physikalischer Konzepte auf die Biologie. Schwerpunkt sind dabei durch schwache elektromagnetische Felder induzierte Resonanz-Effekte in den Zellen von Lebewesen.

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NON LINEAR I.R. EFFECTS IN ACETANILIDE, A MODEL SYSTEM FOR PROTEINS

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Abstract

Measurements of infrared absorption and Raman scattering on crystalline acetanilide, a model system for proteins, show a new band close to the conventional amide I band. Equilibrium properties and spectroscopy data rule out explanation based on conventional assignments. A detailed analysis show that a soliton model, similar to that proposed by Davydov for the α helix in protein, is in satisfactory agreement with the experimental data. Spectroscopy results supporting such assignment are presented and some possible biological implications are briefly mentioned.

An infrared active soliton in a model crystal

Hydrogen bonding is widespread in biomacromolecules, presenting physical features that vary greatly from one case to another. In weak bonds this interaction can be treated as a problem in electrostatics involving a set of fixed and localized interacting charges. On the other hand, in the case of very strong hydrogen bonds, one is faced with a delocalized charge distribution to be treated according to valence theory. Betweeen these two extremes, there are cases where the bonds are of intermediate strength. In these cases, one can model the complex state of affairs by assuming that the local charges depend upon their mutual distances and that these distances in turn depend upon the local charges. Thus in these intermediate cases one can visualize the microscopic source of the anharmonic coupling which gives rise to the non-linear terms in the equations describing the dynamics of the system. This is the case of the N-H ... O=C bond in proteins, for R(NO) distances close to 2.80 A°.

Acetanilide ($CH_3CONHC_6H_5$), or ACN, is an interesting solid because two close chains (spines) of nearly planar hydrogen-bonded amide groups run through the crystal, providing an interesting model for an array of hydrogen-bonded amides in one direction. Moreover, the bond distances in ACN are very close to those found in alpha-helices, where three similar spines are coiled along the helix axis. Since the physical properties of hydrogen-bonded systems are very sensitive to bond distances, we thought ACN would be a useful model system to be used in

searching for new physical features of extended polypeptide chains and perhaps also proteins. We found that a new amide-I band appears at low temperature in crystalline ACN, red-shifted by 15 cm^{-1} from the primary amide-I band at 1665 cm^{-1}, and a large number of other experiments by Raman, X-rays, specific heat and isotropic substitution pointed out that the new band at 1650 cm^{-1} is characteristic of the amide group of ACN in crystal form. A detailed analysis by the usual exiton model cannot account for this new band.

Having excluded conventional explanations we (1), (2), (3), (4) consider the possibility of assigning it to a collective excitation similar to the soliton proposed by Davydov for alpha-helix in proteins (5), (6). Davidov's soliton arises from a cooperative interaction between localized amide-I bond energy and lattice distortion. The bond energy acts through non-linear coupling, as a source of lattice distortion. This lattice distortion reacts, again through non-linear coupling, as a potential well to trap the bond energy and prevent its dispersion via dipole-dipole coupling effects. We followed the same theory with one important difference: for lattice distortion we substituted displacement of the hydrogen-bonded proton. The distinction is vital, because Davydov has shown that photon absorption by his intermolecular vibrational soliton is ruled out by the Frank-Condon principle. Here I shall limit myself to outlining the major points and presenting some conclusions. The main idea was that the effect of introducing localized amide-I energy could displace the ground state of the adjacent hydrogen-bonded proton. This displacement of the proton acts to trap the

amide-I band energy and prevent its dispersion via dipole-dipole interaction effects. The combined excitation was proved to be a soliton, and we assigned the binding energy of this soliton to the experimentally observed red shift of 15 cm^{-1} from the conventional amide-I band to the unconventional amide-I band. A more recent theoretical work (7) has identified this unconventional band as a vibronic analog of a small Holstein polaron.

Biological implications

The work of ACN reported above has been motivated by a few relevant biological implications that we believe it would be appropriate to mention. Biochemical events involving the reactions or changes of state of a macromolecule may be supposed to occur one step at a time (8). Thus they may often be represented in terms of a network of closed pathways or loops. At equilibrium there will be no net circulation around the network, the conditions of detailed balance being fulfilled. A living system requires process, as in the form of a one-way circulation around the network. In the fully-developed organism this state of affairs, which provides for the transduction of free energy from one chemical reaction to another, is made possible by the presence of highly developed polyfunctional enzymes. In an effort to provide a mechanism for this one-way circulation, we have recently introduced the concept of a trapped soliton as an energy packet created at one point of the network and liquidated at another (9). This mechanism can play a role in many biochemical

cycles and perhaps also in prebiotic recations (10). We postulated the formation of a soliton trapped as a ligand on the protein matrix, for which the surrounding heat bath serves as a sink. This soliton is assumed to display physical features quite similar to those observed experimentally in ACN. All this follows from the intrinsic structure of the hydrogen-bonded polypeptide chain itself, because of its capability to let the vibrational soliton be stable or decay in different backbone conformations.

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Energy Transfer in Nonlinear Vibration Systems

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The nonlinear energy channeling among the normal modes of a vibration system is analysed on the basis of the Manley - Rowe energy relations.

Introduction

Frohlich derived a kinetic equation (FKE) describing the basic principles of the excitation of the coherent states and the energy transfer among the normal vibration modes [1 - 5]. The fundamental terms in the FKE contain one- and two-quantum processes representing the energy suply, the energy losses to the heat bath, and the energy channeling between the normal modes with the help of the heat bath. Livshitz and Yushina pointed out the significance of some additional terms in the FKE [6 - 8]. Coherent vibration systems are very likely located in cellular membranes. Due to the membrane potential the vibration systems have very likely highly nonlinear properties. The multiple quantum processes of the energy transfer, therefore, may be significant as is described in [9 - 10].

Manley - Rowe Energy Equations

Manley and Rowe [11] derived the energy relations (MRR) for a nonlinear elecric oscillator with a wide spectrum of the mode frequencies. They assumed the total energy of the oscillator to be constant. The MRR are valid for any nonlinear oscillator [9 - 10]. For a classical nonlinear oscillator the MRR may be easily derived using the Fourier expansion. We get

$$(1)\qquad \sum_{n=-\infty}^{+\infty}\sum_{m=0}^{+\infty}\frac{mP_{m,n}}{m\omega_1 + n\omega_2} = 0\,, \qquad \sum_{m=-\infty}^{+\infty}\sum_{n=0}^{+\infty}\frac{nP_{m,n}}{m\omega_1 + n\omega_2} = 0\,,$$

where $P_{m,n}$ is the derivative of the energy with respect to the time in the mode with the circular frequency $m\omega_1 + n\omega_2$ (m, n are integers).

The quantum anology of Eqs. (1) is derived in [9]. The Hamiltonian of the vibration system may be given as

$$(2)\qquad H = H_0 + H_1 + H_2\,,$$

where H_0 and H_1 are given by [12 - 13]

$$(3) \qquad H_0 = \sum_i \hbar\omega_i a_i^\dagger a_i + \sum_k \hbar\Omega_k c_k^\dagger c_k + \sum_g \hbar\Omega_g P_g^\dagger P_g \ ,$$

$$(4) \qquad H_1 = \sum_{i,k} (\varphi a_i c_k^\dagger + \varphi^* a_i^\dagger c_k) + \sum_{g,i} (\xi P_g a_i^\dagger + \xi^* P_g^\dagger a_i) +$$

$$+ \sum_{i,j,k} (\chi a_i^\dagger a_j c_k^\dagger + \chi^* a_i a_j^\dagger c_k) \ ,$$

and the multiple quantum term H_2 is given by [9 - 10]

$$(5) \qquad H_2 = \sum_{i,j,\ell} \sum_{m,n} [\varkappa a_\ell^\dagger (a_i)^m (a_j^\dagger)^n + \varkappa^* a_\ell (a_i^\dagger)^m (a_j)^n +$$

$$+ \sigma a_\ell^\dagger (a_i^\dagger)^m (a_j)^n + \sigma^* a_\ell (a_i)^m (a_j^\dagger)^n +$$

$$+ \mu a_\ell^\dagger (a_i^\dagger)^m (a_j)^n + \mu^* a_\ell (a_i^\dagger)^m (a_j)^n] \ .$$

Here ω_i, Ω_k, Ω_g are the circular frequencies, $a_i^\dagger$, $c_k^\dagger$, $P_g^\dagger$ are the creation operators, and a_i, c_k, P_g are the anihilation operators of the vibration system, of the heat bath, and of the energy source, respectively; φ, ξ, χ, $\varkappa$, σ, μ are the coupling coefficients. Using the time dependent perturbation theory the MRR may be derived in the form

$$(6) \qquad \sum_{n=-\infty}^{+\infty} \sum_{m=0}^{+\infty} \frac{m\langle \dot{N}_q \rangle \omega_q}{m\omega_i + n\omega_j} = 0 \ , \qquad \sum_{m=-\infty}^{+\infty} \sum_{n=0}^{+\infty} \frac{n\langle \dot{N}_q \rangle \omega_q}{m\omega_i + n\omega_j} = 0 \ ,$$

where $\langle \dot{N}_q \rangle$ is the expectation value of the first derivative (with respect to the time) of the number of the energy quanta in the qth mode. Eqs. (6) represent the interactions between the modes without the help of the heat bath and express the principle of the conservation of the energy.

Nonlinear Energy Channeling

The expectation values $\langle \dot{N}_q \rangle$, $\langle \dot{N}_i \rangle$, $\langle \dot{N}_j \rangle$ are given by

$$(7) \qquad \langle \dot{N}_q \rangle = -B \ , \quad \langle \dot{N}_i \rangle = \sum_{m=0}^{+\infty} \sum_{n=0}^{+\infty} mB \ , \quad \langle \dot{N}_j \rangle = \sum_{m=0}^{+\infty} \sum_{n=0}^{+\infty} nB \ ,$$

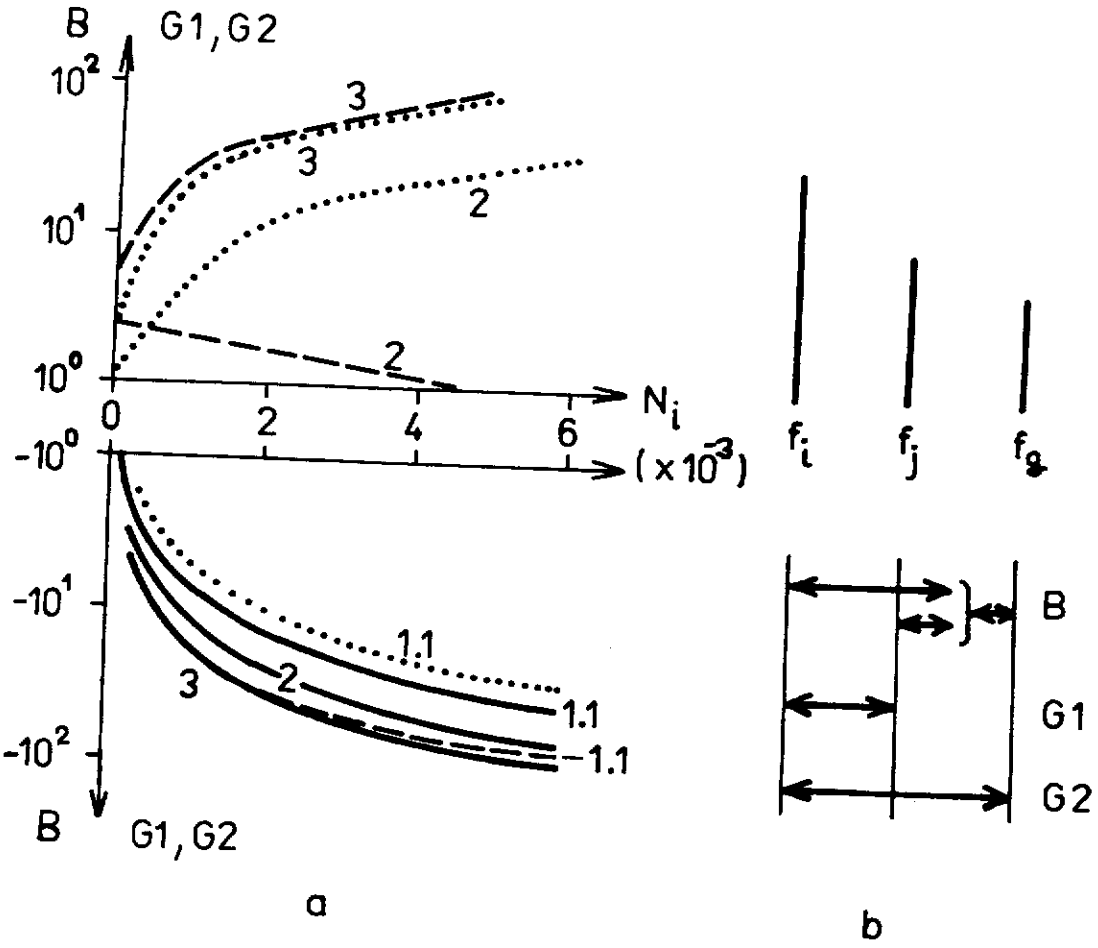

Fig. 1: a) B, G_1 (the dashed curves), and G_2 (the dotted curves) as a function of N_i (the ith mode has the lowest frequency).
b) A schematic picture of the energy channeling among the modes with the frequencies f_i, f_j, and f_q.

where

$$(8) \qquad B = -(2\pi/\hbar)|x|^2[(\langle N_q\rangle + 1)\alpha_i(m)\beta_j(n) - \langle N_q\rangle\beta_j(m)\alpha_j(n)] ,$$

$$\alpha_i(m) = \langle N_i\rangle(\langle N_i\rangle - 1)\ldots(\langle N_i\rangle - m + 1) ,$$

$$\beta_j(n) = (\langle N_j\rangle + n)(\langle N_j\rangle + n - 1)\ldots(\langle N_j\rangle + 1) .$$

Eqs. (7 - 8) are valid for $\omega_q = m\omega_i + n\omega_j$ and represent the x term in the H_2.

Similar equations may be derived for the σ and the μ terms in the H_2. (The σ and the μ terms represent the processes with the frequency relations $\omega_q = -\omega_i + \omega_j$, and $\omega_q = \omega_i - \omega_j$.) These relations describe the energy channeling among the vibration modes the frequencies of which are in convenient relationships.

The direction of the energy channeling depends on the energy excitations of the modes in question. All the equations represent only an approximate solution as the multiple quantum terms with the help of the heat bath were neglected.

We evaluated the B coefficients for a simple model of three interacting modes with the frequencies f_i = .1 THz, f_j = .2 THz, and f_q = .3 THz. The energy levels of the qth mode, and of the jth mode are 1.1, 2, and 3 times greater then the levels in the thermal equilibrium. The nonlinear process in question channels the energy from the ith mode and the jth mode to the qth mode (i.e. upwards) as follows from Fig. 1a. The χ process of the FKE represented by the G_1 and G_2 coefficients (Fig. 1b) channels the energy downwards if the energy levels of the jth mode and of the qth mode are sufficiently high. B, G_1, and G_2 are given in relative values.

Conclusions

1) The nonlinear energy transfer among the normal modes of a vibration system includes the multiple quantum processes with the help of the heat bath and those without the help of the heat bath. We analysed the processes without the help of the heat bath.

2) The direction of the energy flow may be downwards as well as upwards. The direction of the energy flow depends on the energy levels and on the frequencies of the vibration modes.

3) The electromagnetic field generated by the coherent vibrations in living cells has very likely a fundamental biological function. The changes of the field may be connected with the nonlinear interactions.

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RESUME DE LA COMMUNICATION "L'ORDRE CHAOTIQUE"

par P. Bergé

Le but de la communication est de montrer que le comportement très compliqué d'un système n'est pas nécessairement à rattacher à une complexité inhérente à ce dernier.

Il est naturel d'associer une évolution erratique, imprévisible, à la conjonction d'une multiplicité d'évènements indépendants. C'est ainsi que les numéros que l'on tire au loto sortent de manière parfaitement imprévisible -erratique donc- du fait du très grand nombre de chocs que subissent les boules avant d'être extraites. Il en est ainsi de beaucoup de phénomènes dont la nature aléatoire est à relier à une loi des grands nombres : en effet, une quantité dont la grandeur résulte de l'effet d'une multitude de variables indépendantes aura nécessairement une variation irrégulière.

A l'opposé, les mathématiques nous donnent des exemples d'équations très simples dont la solution est, néanmoins, parfaitement irrégulière. C'est ainsi qu'on peut tirer des nombres au hasard, aussi efficacement qu'à la loterie, par une transformation simple que l'on recommence répétitivement. Par exemple, on prend un nombre X_0 entre 0 et 1 (pas trop simple !) ; on calcule l'image X_1 de ce nombre par la relation $X_1 = 4\ X_0\ (1 - X_0)$ puis on calcule l'image X_2 de X_1 par la même relation et ainsi de suite.

On obtient ainsi une série de nombres X_0, X_1, X_2 qui se succèdent au hasard, c'est-à-dire sans que l'on puisse déterminer un ordre dans leur apparition (on dit que la série est chaotique). Et pourtant, quoi de plus déterministe que la relation qui les engendre ? C'est ce paradoxe (apparent) qui traduit un nouveau concept : celui de chaos déterministe. Quittant les mathématiques et abordant les systèmes "réels", nous pouvons dire que point n'est besoin d'une complexité inhérente au système pour que son évolution soit erratique. C'est ainsi que les systèmes les plus simples peuvent avoir des comportements chaotiques, ce qui repose le problème plus philosophique de la vraie nature du hasard.

.../....

Dans ce cas, le comportement chaotique, c'est-à-dire l'impossibilité de faire des prévisions, provient d'une amplification incessante des plus infimes incertitudes dans les conditions initiales (S.C.I.). Entendons par là que si nous considérons deux conditions initiales extrêmement voisines, l'écart minime qui les sépare initialement va augmenter sans cesse (exponentiellement) quand le temps passe. C'est ainsi que les deux évolutions correspondantes, un moment semblables, vont devenir rapidement très différentes. C'est ce qui interdit, en pratique, toute prévision dans un système doté de SCI. Cette SCI peut intervenir dès que des non-linéarités sont présentes et que le nombre de variables du système (appelées degrés de liberté) est au moins égal à 3. C'est ainsi qu'un pendule sollicité périodiquement peut présenter des mouvements chaotiques.

On comprend toute l'importance de ce nouveau concept qui peut remettre en question bien des interprétations et des idées reçues. Citons, par exemple, l'évolution fort désordonnée de la concentration dans certaines réactions chimiques particulières. On aurait pu les attribuer à une mauvaise stabilisation de l'un des paramètres entraînant la présence de fluctuations. En fait, il n'en est rien et on a pu montrer que ce "chaos chimique" résultait de l'évolution déterministe d'une simple oscillation de la réaction. Beaucoup de phénomènes, incompréhensibles de par leur comportement erratique, ont peut-être une origine fort simple et déterministe...

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NONLINEAR ELECTRODYNAMICS IN CELL MEMBRANE TRANSDUCTIVE COUPLING OF ELECTROMAGNETIC FIELDS AND HUMORAL STIMULI

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A connected picture of the sequence and energetics of major events that couple humoral stimuli (hormones, neurotransmitters, antibodies) from surface receptor sites to the cell interior is now emerging from use of weak electromagnetic (EM) fields to manipulate inward and outward signal streams through cell membranes.

1. Intramembranous Protein Particles (IMPs) and the Fluid Mosaic Cell Membrane Model."

There has been a remarkable growth in knowledge of the structural and functional organization of cell membranes through use of other sophisticated research tools. On the one hand, these studies have revealed numerous strands of protein (intramembranous particles, IMPs) inserted into the thin double layer of fat molecules that forms the plasma membrane. On the other, studies of both electrical and chemical intercellular communication have focused on specialized regions of contact between adjacent cell membranes. These regions form gap-junctions that couple cells electrically and chemically.

IMPs span the membrane from inside to outside. They have external protrusions into the fluid surrounding the cell. Their outer tips are terminal glycoprotein strands that sense electric fields and form receptor sites for chemical stimulating molecules. They have functional contacts inside cells with key elements of the cell machinery, including enzymes and the numerous fine tubes of the cytoskeleton. They make functional contact inside the cell with key elements of the cell machinery, including enzymes and the numerous fine tubes of the cytoskeleton. IMPs "float" in the sea of lipid molecules of the plasma membrane, leading to the generally accepted "fluid mosaic model of the cell membrane (Singer and Nicolson, 1972). Thus, these intramembranous strands form signaling pathways by which external stimuli are sensed and conveyed to the cell interior.

2. Intercellular Communication Through Gap Junctions.

The regions of contact between membranes of adjacent cells form gap junctions that couple cells electrically and chemically (Robertson, 1963). These junctions are perforated by numerous tiny tubes (connexons) that span the entire membrane that allow ionic coupling and metabolic cooperation in transfer of essential metabolic substances between cells (Fletcher et al., 1987). Disruption of intercellular communication through gap junctions leads to serious disorders of growth control, including tissue repair and neoplastic transformation (Loewenstein, 1981). Our studies indicate a synergic action of chemical cancer promoters with weak EM fields in modification of gap junction functions. Disruption of gap junctions is now viewed as a prime factor in cancer promotion and tumor formation (Adey, 1988a and b; Trosko, 1987; Yamasaki, 1987).

3. Electrical Benchmarks in Physiological Organization of Cells and Tissues.

The membrane potential of approximately 0.1 V exists across the extremely thin lipid plasma membrane, typically about 40° thick; a membrane so thin that in consequence there is an enormous electric gradient of 10^5V/cm across the cell membrane. Physiological electric oscillations in fluid surrounding cells are many orders of magnitude weaker than this natural barrier of the membrane potential. These weak pericellular gradients have therefore been considered too weak to play a physiological role.

Nevertheless, many organisms including man are sensitive to tissue gradients in the range 0.1-100 mV/cm. These sensitivities have been observed in many tissues and cell cultures, including lymphocytes, and cells from liver, ovary, skin, bone, cartilage and nervous tissue (see Adey, 1981, 1984, 1988a and b for reviews).

These interactions require appropriate consideration of the role of cooperative processes and associated nonlinear electrodynamics at cell membranes revealed with imposed EM fields (Adey and Lawrence, 1984). These cooperative phenomena are in the realm of nonequilibrium thermodynamics, far removed from traditional equilibrium models of cellular excitation that have focused on depolarization of the membrane potential and on associated massive changes in ionic equilibria across the cell membrane.

3. Cooperative Processes Initiated by EM Fields at Cell Surfaces, with Amplification of Initial Signals.

Extracellular current flow in tissue associated with physiological activity and with imposed EM fields spreads longitudinally through narrow gutters separating adjacent cells. Initial events associated with these pericellular fields and with binding of stimulating molecules at their receptor sites elicit a highly cooperative modification of Ca^{2+} binding to glycoproteins along the membrane surface. This is an amplifying stage. From concurrent manipulation of initial molecular binding events with imposed EM fields, there is evidence of a far greater Ca^{2+} efflux than is accounted for in the events of receptor-ligand binding.

Furthermore, there is striking evidence for the nonequilibrium character of this modification in Ca^{2+} binding in its occurrence in quite narrow windows in stimulus frequency and amplitude (Adey, 1988b). "Tuning curves" of altered Ca^{2+} efflux from tissues as a function low frequencies in imposed fields were first described by Bawin et al. (1975, 1976) with maximum sensitivities around 16 Hz. Neither size nor geometry of the test biota are primary determinants of these interactions. Fields in the same frequency range (1-100 Hz) also show intensity windows for Ca^{2+} efflux with induced tissue electric gradients typically in the range 10^{-7}-10^{-1} V/cm. Windowing in frequency, amplitude and time also occurs in a wide range of Ca-dependent cell surface and intracellular mechanisms, including allogeneic cytotoxicity and enzymatic responses to EM field interactions at cell surfaces.

Cooperativity found in these functional linkages in biological systems may be defined as ways in which components of a macromolecule, or a system of macromolecules, act together to switch from one stable state of a molecule to another. These joint actions frequently involve phase transitions, hysteresis, and avalanche effects in input-output

relationships. Initial triggers to cooperative processes may be weak and the amplified responses orders of magnitude larger, raising questions about thresholds and the minimum size of an effective triggering stimulus. Most important is the thermal Boltzmann (kT) noise in the system. At room temperature, this is 0.02 eV and is the basis for molecular collisional interactions. The sensing of a gradient of 10^{-7} V/cm modeled on this threshold would require a cooperative molecular system extending over 300 m. The abundant evidence that extracellular gradients from 10^{-1} V/cm down to this level are biologically significant in systems of cellular dimensions is a strong reminder of the importance of better understanding molecular and morphological substrates of this transductive coupling.

4. Three Stages in Transmembrane Signaling from Cell Surface to Interior

There is a minimal sequence of three steps in transductive coupling between cell surfaces and intracellular enzymatic systems and organelles. Each step is Ca-dependent. a) The first weak electrochemical events at molecular receptor sites and in detection of EM fields are sensed by cell surface glycoproteins. b) These amplified surface events are signaled to the cell interior along transmembrane portions of IMPs. c) Internally there is signal coupling to intracellular enzymes and through the cytoskeleton to the nucleus and other organelles.

The long strands of membrane receptor proteins for the human epidermal growth factor (EGF), the nerve growth factor (NGF) and the insulin receptor have been studied as models of coupling proteins in transmembrane signaling. In all three, the molecule appears to cross the membrane only once. The intramembranous segment is extremely short, and is composed of 23 predominantly hydrophobic amino acids, probably incapable of supporting ionic or protonic conduction. Nevertheless, addition of EGF to human epidermal cell cultures causes a 2-4-fold increase in cytoplasmic free Ca within 30 sec, all derived from extracellular sources and without a change in membrane potential Moolenaar et al., 1986; Ullrich et al., 1985).

We have hypothesized that this transmembrane signaling may involve nonlinear vibration modes in helical proteins, leading to Davydov soliton waves (Davydov, 1979; Scott, 1985). Although evidence for soliton waves in DNA and helical proteins remains inconclusive, studies of millimeter microwave absorption in macromolecules, bacteria and simple cellular systems have revealed highly nonlinear absorption in the frequency range 10-100 GHz (see Adey, 1988b and c for reviews).

5. Molecular Markers of Transductive Coupling Through Cell Membranes: Cytotoxicity Studies and Intracellular Enzyme Activities.

Is cooperative modulation of cell surface Ca binding a first step that culminates in Ca-dependent physiological responses in key enzyme systems within cells?

We have shown that cell membrane mechanisms that mediate cytotoxic destruction of target cells by direct contact (allogeneic T lymphocytes targeted against lymphoma cells, for example) are modulated by weak pericellular EM fields. Three groups of intracellular enzymes respond to signals initiated at cell membranes as a response to EM field exposure: a) membrane-bound adenylate cyclase involved in activation of protein kinases through conversion of ATP to cAMP; b) cAMP-independent protein kinases that perform

messenger functions (Fig. 1); c) ornithine decarboxylase (ODC), essential for growth in all cells by its participation in synthesis of polyamines essential for DNA formation.

6. Models of Cooperative Organization in Physiological Systems.

Experiments cited above imply that interactions occurring at athermal levels between biological substrates and low frequency components of EM fields must take place in biomolecular systems exhibiting dynamic patterns of organization. Historically, these patterns have been studied in populations of elements in terms of complex flow patterns. They can undergo sudden transitions to new self-maintaining arrangements that are relatively stable over time. They are dissipative in nature, since they are sustained by continuous energy inputs and are thus far from equilibrium with respect to at least one important system parameter. As nonequilibrium processes, they may be exhibit resonant or windowed phenomena, an important aspect of their occurrence in tissue interactions with EM fields.

Self-sustained oscillations in biological systems have also been modeled on the requirement for interaction of regular external perturbations with internal oscillations, thus synchronizing the system to the external drives (entrainment). There is a sharp frequency response, exhibiting both frequency and intensity windows and rather irregular behavior near the entrainment region. A further increase in energy of external driving fields, both static and periodic, leads to sequences of period-doubling bifurcations, alternating with quasi-periodic and irregular regions (chaos). As a consequence, a regularly driven self-oscillating system may exhibit intrinsic chaotic behavior, even though the underlying dynamic is strongly deterministic (Kaiser, 1984). Finally, still higher levels of energy input destabilize the system (collapse), leading to the onset of propagating pulses (solitons). A nonlinear temporal structure is thus replaced by a nonlinear spatiotemporal structure in a dispersive process.

7. Summary

Use of weak EM fields to study the sequence and energetics of events that couple stimuli from surface receptor sites to the cell interior has identified the cell membrane as a primary site of interactions with these low frequency fields. Field modulation of cell surface chemical events indicates a major amplification of initial weak triggers associated with binding of hormones, antibodies and neurotransmitters to their specific binding sites. Calcium ions play a key role in this stimulus amplification.

It is at the atomic level, rather than the molecular, that physical, rather than chemical events now appear to shape the flow of signals and transmission of energy in biomolecular systems. These recent observations have opened doors to new concepts of communication between cells as they whisper together across barriers of cell membranes.

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MARDI 5 JUILLET 1988

9 h - 12 h 30

ETUDES NOUVELLES SUR LES STRUCTURES ET INTERACTIONS MACROMOLECULAIRES

NEW STUDIES OF MACROMOLECULAR STRUCTURES AND INTERACTIONS

SELF-CATALYSIS AND ENZYMATIC ACTIVITY IN RNA. Thomas R. Cech, Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80309-0215, U.S.A.

Chemical reactions in a living cell rarely take place by themselves, but are generally catalyzed by macromolecules called enzymes. It was long thought that all enzymes were proteins. The finding that RNA (ribonucleic acid), a form of the genetic material, can in some cases act as an enzyme overturns this principle, and may throw new light on the origin of life.

Self-catalyzed RNA Splicing.

Eukaryotic genes are often interrupted by stretches of non-coding DNA called intervening sequences (IVS) or introns. RNA polymerases transcribe both the exons (coding sequences) and the intervening sequences to give large precursor RNAs. The intervening sequences are subsequently removed by a cleavage-ligation process known as RNA splicing.

In the case of a pre-ribosomal RNA from *Tetrahymena*, a ciliated protozoan, we found that accurate RNA splicing occurs *in vitro* in the absence of protein (1,2). Splicing requires only a divalent cation (Mg^{2+} or Mn^{2+}), a monovalent cation, and some form of the nucleoside guanosine. The splicing activity is intrinsic to the IVS RNA. RNA self-splicing exemplifies intramolecular catalysis in that specific cleavage-ligation reactions are accelerated far beyond the basal uncatalyzed rate (> 10^{10}-fold).

Tetrahymena pre-rRNA splicing is a two-step mechanism (3). Both steps occur by transesterification, an exchange of phosphate esters that produces no net change in the number of ester linkages. This mechanism explains how RNA ligation can take place without an external energy source as is often provided by ATP or GTP hydrolysis. In the first transesterification, the 3'-hydroxyl of a free guanosine molecule (GTP, GDP, GMP, or guanosine) acts as the nucleophile, attacking the 5' splice site (Fig. 1, top). This reaction leaves a 3' hydroxyl group at the end of the 5' exon, which can then act as the nucleophile for the second transesterification reaction, exon ligation.

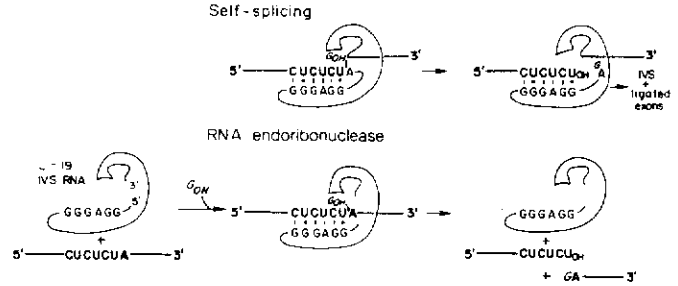

Fig. 1. A model for the endonuclease action of L - 19 IVS RNA. The mechanism is an intermolecular version of the first step of pre-rRNA self-splicing. Thin letters and lines, IVS sequences; bold letters and thick lines, exon sequences (top) or substrate RNA sequences (bottom); the G in italics is a free guanosine nucleotide or nucleoside.

Self-splicing has also been demonstrated for a number of other rRNA precursors and mRNA precursors from fungal mitochondria and bacteriophage T4 as well as nuclei (reviewed in ref. 3). In many cases these undergo self-splicing by the same guanosine-dependent transesterification mechanism described for *Tetrahymena* nuclear pre-rRNA splicing; in other cases self-splicing occurs by a lariat-formation mechanism analogous to that described for nuclear pre-mRNA splicing.

The *Tetrahymena* IVS RNA as an Enzyme

After its excision from the *Tetrahymena* pre-rRNA, the IVS RNA retains catalytic activity. For example, shortened forms of the IVS RNA, or "ribozyme", can perform a variety of cleavage-ligation reactions with oligoribonucleotide substrates (4,5). These include nucleotidyl transfer reactions that allow the ribozyme to function as a sequence-specific endoribonuclease (Fig. 1, bottom). The ribozyme works with multiple turnover, thereby satisfying the definition of an enzyme. The sequence specificity of these reactions can be altered in a predictable manner by site-specific mutagenesis of the active site of the ribozyme (5).

The ribozyme can also catalyze RNA polymerization reactions (4,6). For example, pentacytidylic acid (C_5) can be extended by the successive addition of mononucleotides derived from a guanylyl-(3'-5')-nucleotide (GpN). C's or U's are added to C_5 to generate chain lengths of 10 to 11 nucleotides, with longer products being generated at greatly reduced efficiency (6). The reaction is analogous to that catalyzed by a replicase with C_5 acting as the primer, GpN's as the nucleoside triphosphates, and a sequence in the IVS providing a template. The demonstration that an RNA enzyme can catalyze net elongation of an RNA primer supports theories of prebiotic RNA self-replication.

Structure of the Active Site for RNA Catalysis.

Some of the sequences and structures of the IVS that are involved in substrate sequence specificity and in catalysis have been identified. A tertiary-structure model of the active site of this RNA enzyme has been constructed (Fig. 2) based on comparative sequence analysis of related group I intervening sequences (7,8), data on the accessibility of each nucleotide to chemical and enzymatic probes, and principles of RNA folding derived from a consideration of the structure of tRNA determined by X-ray crystallography (9).

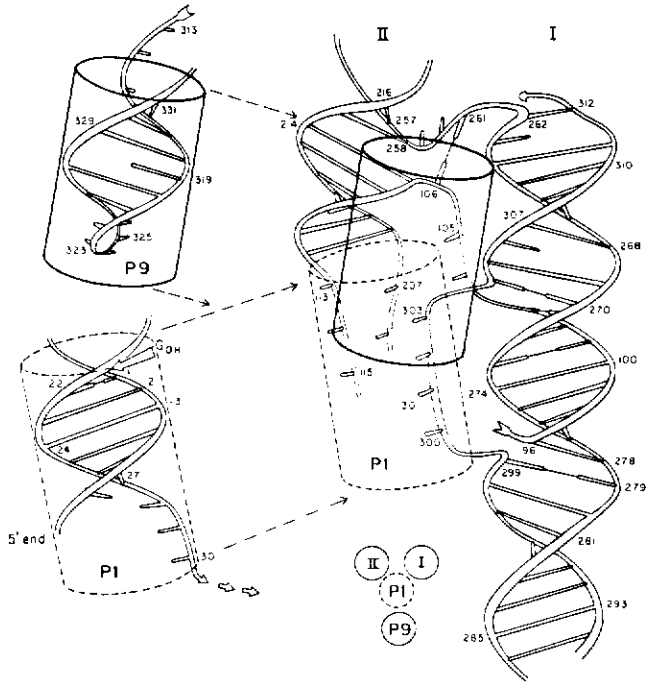

Fig. 2. Three-dimensional structure model of the active site of a ribozyme. Possible interaction of the 5' splice-site duplex (P1, dashed cylinder) with the catalytic center of the *Tetrahymena* IVS RNA is shown. Paired element P9 (solid cylinder) might interact with P1 from the side opposite that of domains I and II of the catalytic center as shown. Small diagram at bottom depicts top view of the model. See Kim and Cech (9) for details.

In the model, the catalytic center has a two-helix structural framework composed of the base-paired segments of the group I conserved sequence elements. The structural framework supports and orients the conserved nucleotides that are adjacent to the base-paired sequence elements; these conserved nucleotides are proposed to form the active site and to bind the 5' splice-site duplex and the guanine nucleotide substrate (Fig. 2). Tests of the model are in progress.

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GENETIC ANALYSIS OF SMALL NUCLEAR RIBONUCLEOPROTEIN PARTICLES (snRNPs) IN YEAST. Christine Guthrie, Department of Biochemistry and Biophysics
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The process by which introns are removed from precursors to messenger RNA (pre-mRNA) in eukaryotic nuclei shares certain fundamental properties with splicing reactions of the group II type (1). Since the latter can occur autocatalytically in vitro, catalysis must be achieved by the formation of active sites comprised solely of RNA. Highly conserved structural elements which reside primarily within the intron have been shown to underly the geometry required for function. In contrast, the only conserved features of nuclear pre-mRNA introns are restricted to short regions at or near the splice junctions, and splicing takes place in association with an extensive trans-acting machinery dubbed the spliceosome. Work during the last several years has revealed that prominent trans-acting factors are U1, U2, U4, U5 and U6, five small nuclear RNAs (snRNAs) which exist as ribonucleoprotein complexes termed snRNPs (2). Presumably the low information content in cis within this class of introns is compensated by the participation of the snRNPs, which act in trans to impart the appropriate architecture for catalysis.

In principle, both RNA- and protein-based interactions contribute to communications between the snRNPs and the intron and the snRNPs with one another. In cognizance of group II reactions, however, it seems evident that nuclear pre-mRNA splicing evolved from an RNA machine. As with the recent revolution in our understanding of the ribosome, the mechanism may be most directly appreciated by seeking to establish the RNA-mediated aspects of the reaction. We have thus chosen to focus our efforts on the snRNA components of the spliceosomal snRNPs. In particular, we have carried out our studies on the snRNAs from the budding yeast Saccharomyces cerevisiae because of the ability to exploit the facile genetic techniques available in this organism. Moreover, this has allowed us to obtain information on snRNA structure from a broad phylogenetic distance. The power of comparative sequence analysis to identify functionally important features of sequence and structure has been clearly demonstrated in the tour-de-force studies of rRNA pioneered by Woese, Noller and their colleagues (3).

Our knowledge of the complete set of spliceosomal snRNAs from yeast has provided several unexpected insights into the evolutionary constraints operating on the five snRNA species. Interestingly, the similarity between the yeast and mammalian homologues varies considerably. The structure of yeast U2 RNA is quite surprising: the S. cerevisiae RNA is more than six times as long as its human counterpart (1,175 vs. 189 ntes.) and almost all the sequence similarity is in the first 80 nucleotides, where the two RNAs are 75% identical (4). Likewise, yeast U5 (5) and U1 (6) RNAs are respectively 1.5 and 3.5 times the length of their mammalian counterparts, with which they share only limited sequence and structural similarity. In contrast to the other spliceosomal RNAs, however, U4 and U6 are very close in size to those in mammals. The primary structure of yeast and human U4 is significally different. However, yeast U6 is virtually identical in size,

sequence and structure to its mammalian homologue (7). These apparent constraints argue for a central role in the splicing process and suggest that U6 snRNA is in close contact with several components of the machinery.

Each of the yeast snRNAs is encoded by a single copy gene (in striking contrast to the large multi-gene familes characteristic of snRNAs in higher eukaryotes) and we have shown, using the technique of one-step gene replacement, that the genes are essential for growth (4, 5, 7). To prove that these snRNAs are required for splicing in vivo, we have engineered their conditional synthesis, by fusing the coding sequences to a promoter whose activity can be controlled in response to carbon source (GAL1); thus, by shifting cells from galactose to glucose, we can inhibit further expression from this essential gene and observe the accumulation of unspliced pre-mRNAs as the snRNA is diluted out during subsequent cell divisions (5). With these tools in hand, we are now attempting to understand how the RNAs mediate the splicing reaction.

The process of splicing can be broken down into several types of recognition events: 1) interactions between signals in the substrate and trans-acting factors, and 2) the coalescence of these factor-site assemblies into a higher order structure, the mature spliceosome. The archetype of snRNA-mediated recognition events is the interaction between U1 and the 5' splice site. Steitz and colleagues first proposed that this occurs by complementarity between the 5' splice junction and highly conserved sequences at the 5' terminus of U1 (8). We have recently subjected this model to direct genetic tests by the creation of base changes in U1 predicted to compensate for 5' splice site mutations which inhibit the reaction. These experiments utilize a chimeric splicing substrate which has an easily assayable phenotype: proper splicing of an actin-HIS4 fusion allows cells to grow on media containing the histidine precursor histidinol (Hol). Thus, we can test the hypothetical base-pairing by asking if cells containing a fusion with 5' splice site mutations (and are thus Hol-) regain the ability to grow on Hol after transformation with a plasmid carrying an appropriately engineered snRNA gene. This biological suppression assay is followed by biochemical analyses which directly test whether splicing of the mutant fusion is restored. In this way we have demonstrated base-pairing interactions between U1 and the 5' splice site and between U2 and sequences upstream of the 3' splice site (9). Interestingly, the latter interaction appears to mimic a structure known to be essential for autocatalytic group II splicing, an intermolecular helix containing a "bulged" adenosine residue.

Our understanding of the snRNP-snRNP interactions which mediate the formation of higher order structure is currently quite limited. By far the best understood interaction between snRNP components is the association between U4 and U6, which are ordinarily isolated together in a single snRNP particle. We have obtained recent phylogenetic data that allow us to describe an extensive base pairing interaction between the two molecules (7). This interaction, which confers a high temperature stability (Tm ~53 degrees C), is of particular interest in light of the unexpected finding that spliceosomal complexes about to undergo the

first nucleolytic cleavage event have lost U4, but retain U6. Thus the association between U4 and U6 is dynamic and requires a substantial structural rearrangement. We are now trying to understand the mechanism which mediates this long sought-after example of an RNA "conformational switch." Ongoing mutational analysis of the U4 and U6 RNAs should shed light on this fascinating process.

Finally, we are using genetics to establish which structural features of the snRNAs serve as recognition sites for binding of snRNP proteins. One approach to this problem is the isolation of extragenic (i.e., second site) suppressors of snRNA mutations. For example, a set of polypeptides which associate with each of the splicoeosomal snRNAs, termed the "Sm" proteins, are known to bind to a consensus sequence rich in pyrimidines. Mutations in this element confer a lethal phenotype; rare viable cells may arise by the selection of mutations in the Sm proteins which allow them to recognize the altered site (or, simply to bind with higher affinity to all sites). We have validated the efficacy of this general type of approach by isolating a suppressor of a mutant intron. The mutated locus encodes a dominant, trans-acting, allele-specific suppressor. These and other properties argue that we have identified a structural gene encoding a component of the splicing machinery that physically interacts with the intron (10). We have recently cloned the suppressor gene and shown that the wild-type locus is essential for viability. Sequence analysis and genetic mapping are underway; together with the generation of antibodies to the gene product, and of conditionally lethal point mutations within the gene, these experiments should uncover the structure and function of this essential splicing factor.

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MARDI 5 JUILLET 1988

14 h 30 - 18 h

METHODES NOUVELLES D'ANALYSE DES STRUCTURES

NEW STRUCTURAL METHODS

Opening remarks on "New Structural Methods"
Conference Institut de la Vie
July 1988

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Current progress in the elucidation of protein function relys on our knowledge of protein structure as being continuously produced by molecular cloning of DNA sequencing, by protein isolation and sequencing technique, by model folding analysis and essentially by multiple studies of crystaline proteins, soluble and membrane bound via their X-ray deflection patterns.

In recent years, this type of experimentation yielded in general highly resolved structures at the atomic level of a large number of proteins. In addition, the delicate and complex motion of individual goups within the protein has been recognized indicating intrinsic functional properties of protein molecules.

A large number of physical techniques are currently in use and developed in order to complement the static picture of proteins which we gained by X-ray deflection analysis, and to focus on protein function. Two-dimensional NMR, EXAFS, ESR are topics of our discussion here today. However, I would like to point to the rapidly developing infrared spectroscopy, which strongly enhances our current understanding of intramolecular processes occurring in proteins. Indeed, time-resolved FTIR yielded new insights into membrane molecules such as bacteriorhodopsin or the photochemical reaction center of bacteria from which additional information is gained on localized events in proteins.

"Macromolecular crystallography and synchrotron X-radiation"

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Introduction

The development of new applications of synchrotron radiation to protein crystallography rests on an improved understanding of diffraction physics and the harnessing of new technical developments including instrumentation computing, and molecular genetics. I will address three areas in my talk:- Laue techniques, diffuse scattering from single crystals and anomalous dispersion.

A. The Laue method

Rapid data collection via monochromatic methods has been surpassed by a return to the Laue method[1]. The consideration of the Laue method for quantitative structure determination had several fundamental obstacles arguing against it. These arguments were (i) the multiplicity or overlapping orders problem[2],[3]; (ii) the wavelength normalisation problem[4]; and (iii) could a protein crystal sample withstand the radiation blast of the beam incident upon it[5],[6]. The application of rapid data collection methods (with monochromatic or white beams) to time resolved crystallography (ref[7]) has various other problems to be resolved involving the initiation of events in a crystal and development of several conformational substates in a particular system.

(i) Multiplicity problem

According to W L Bragg[2] "X-ray analysis started with the Laue photograph. It is too hard to attach a quantitative significance to the intensity of the spots, which are due to the superposition of diffracted beams of several orders selected from a range of 'white' radiation." and to R W G Wyckoff[3] "In general, reflections to be used in establishing the structure of a crystal should involve wavelengths between the low wavelength limit, λ_{min} and $2\lambda_{min}$. Otherwise if $n\lambda > 2\lambda_{min}$ an observed reflection may be partly of one and partly of another order. Only when it is known in some other way that planes of particular types do not give reflections in the first one or more orders can higher values of $n\lambda$ be safely employed in intensity comparisons."

Analysis of pea lectin Laue patterns recorded on the SRS wiggler started with a prediction from the experimental parameters and the Laue geometry (polychromatic beam at normal incidence to a flat film). This revealed the wavelength, spatial and multiplicity distributions of these patterns[8],[9]. It appeared that the Laue spots were not generally multiple but were in fact mainly single reflection, single wavelength. Theoretical work provided the explanation for this[10]; this involved analysing the probability that an integer triplet (hkℓ) could have a common integer divisor and combining this probability with volume elements of reciprocal space. Moreover, a probability map was generated indicating those regions of reciprocal space that would be primarily recorded as single. Finally, the close agreement of this processed, wavelength normalised pea lectin Laue data with pea lectin oscillation/monochromatic data confirmed that the theory and the dominance of singles was correct.

The objections of Bragg and Wyckoff are fundamental in the sense that an energy sensitive detector with the additional properties of high spatial resolution, high count rate and high absorption efficiency is not available. The freeing of the need for energy resolution is a major relaxation of the detector requirement. In contrast, the spatial overlap problem[5],[9] is at least tractable by a number of approaches. These include increasing the film size. Alternatively spatial deconvolution methods can be applied (Greenhough pers comm)

(ii) Wavelength normalisation

According to Bragg[4] "The deduction of the crystal structure from the appearance of the Laue photograph is a complicated process, because the intensities of the spots do not depend upon the structure alone. They depend also upon the strength of the components in the continuous range of the original beam to which they are respectively due, and each spot may be composed of several orders superimposed. They are also influenced by the different blackening effect of radiation of different wavelength, and complications arise here owing to the absorption of the X-rays by the silver and bromine in the photographic plate. In spite of these difficulties, the Laue photograph can be made a sound method of analysis, and has, for instance, been used with striking success by Wyckoff. Advantageous features are the ease and certainty with which indices can be assigned to the spots, and the wealth of information represented by a single photograph. Nevertheless, the methods which employ monochromatic radiations are more direct and powerful."

Each reciprocal lattice point is stimulated by some wavelength within the experimental bandwidth. A variety of wavelength dependent factors affect the measured structure amplitude. These factors include the SRS spectral profile, the effect of optical elements, sample scattering efficiency, absorption of components in the beam and film response.

In the quantitative analysis of Laue patterns the correction of the Laue data to a standard wavelength for comparison with "standard" monochromatic data requires explicit wavelength normalisation. Such a normalisation can be achieved with the Laue data alone using equivalent reflections recorded at different wavelengths (Campbell *et al* (1986)) - it is therefore independent of monochromatic data and free of bias. Comparison of the wavelength normalised protein crystal Laue data with monochromatic data reveals that there are no systematic errors due to multiple scattering, for example, (as suggested by Glazer (pers comm)). Other wavelength normalisation methods have been suggested[1] and used[7] which rely on monochromatic data to make the correction. Our goal has been to make independent data comparisons to ascertain the presence of systematic errors. Such errors do not seem to exist. However, absorption corrections for the sample in Laue geometry have yet to be implemented.

(iii) Sample behaviour in the beam:pea lectin

Radiation damage of protein crystals and synchrotron radiation has always been a worry[11]. The behaviour of samples in monochromatic beams has not, generally, been found to be a serious problem. In the case of the white beam the intensity is strong enough that absorbed radiation may generate a heating problem. Pea lectin crystals are probably the most radiation insensitive crystal known. They were therefore adopted at Daresbury for detector calibration trials. These crystals were obviously ideal also to test the Laue method and to see if they could withstand the white beam. It turned out that five exposures of the white beam could be recorded from a single position of the crystal. A further two shots showed increasing mosaic spread (and spot size) before the crystal no longer diffracted. Analysis of these data revealed that very similar quality (I/σ) data were recorded in a time

period two orders of magnitude quicker than monochromatic SR methods[12].

B. Diffuse Scattering

Intense synchrotron radiation is used to measure very high resolution protein crystal data for model refinements. Oscillation film data from a wide variety of samples revealed quite clearly a wealth of diffuse scattering data. These diffuse distributions appear to be different from system to system. Hence it is possible that unique information might be derived from this data about the conformational flexibility of these molecules. Moreover, because of ideas such as hinge bending between protein domains it is interesting to investigate whether the diffuse scattering can be related to intra-molecular protein flexibility.

The understanding of the distribution of the diffuse scattering requires a careful dissection of the contributing components to the background pattern underlying the single crystal Bragg peaks. Radial distribution measurements have been made on a variety of materials.

The acoustic scattering is derived from long range (many unit cells) correlated motion within the crystal; this gives diffuse haloes around the Bragg peaks of an intensity proportional to the Bragg peak intensity. Measurements on the speed of sound have been made to investigate the form of the acoustic scattering. The method used was laser generated ultrasound and involved crystals of ribonuclease and haemoglobin[13]. A value of 1791 ± 67 ms^{-1} was obtained from several crystal orientations on the two sample types. The standard deviation indicates the small degree of anisotropy of the measurements.

C. Anomalous dispersion

The use of multiple wavelengths and synchrotron radiation has now been successfully used for

a) the location of metal atoms

b) the identification of respective metal atoms of near identical atomic number (eg Mn versus calcium in pea lectin)

c) multi-λ phase determination

Several classes of problem have been approached based on the metal type

a) the transition metals (eg K edges of Mn, Fe, Co, Ni, Cu, Zn)

b) heavy atom derivatives (eg L edges of Pt, Au, Hg)

c) the use of selenium to replace sulphur in methionine and thereby utilise the selenium K edge.

In the case of seleno-methionine prepared protein crystals the number of seleno sites in the asymmetric unit of the crystal is likely to be large. This will often make the various anomalous Patterson syntheses complicated to interpret. At York we have been investigating the use of direct methods to locate metal atoms from the anomalous dispersion data at one wavelength and several wavelengths. This work is in collaboration with Fan Hai-fu and his group in Beijing[14].

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Figure 1

Multiplicity distribution of reflections in Laue diffraction. The plot shows the percentage of reciprocal lattice points on the Laue pattern that are single wavelength, single reflecting plane spots as well as the percentage that are double and triple.

(From ref.10)

The Laue patterns are dominated by single component spots in contradiction to the statements by Bragg (See refs 2 and 4) and Wycoff (ref 3).

Figure 2

Laue diffraction pattern of pea lectin.

(From ref.9)

Figure 3

Wavelength normalization curve for pea lectin Laue data.

(From ref.12)

Figure 4

Protein crystal diffuse scattering; concanavalin A as an example. The resolution limit at the edge of the film is 1.4Å.

Figure 5

The location and identification of the Mn and Ca metal cofactors in pea lectin using anomalous dispersion and synchrotron radiation.

Figure 6

A view of a monomer of concanavalin A seen from behind the β sheet. The metal cofactor atoms (Mn and Ca) are shown stippled.

Con-A is structurally homologous to pea lectin.

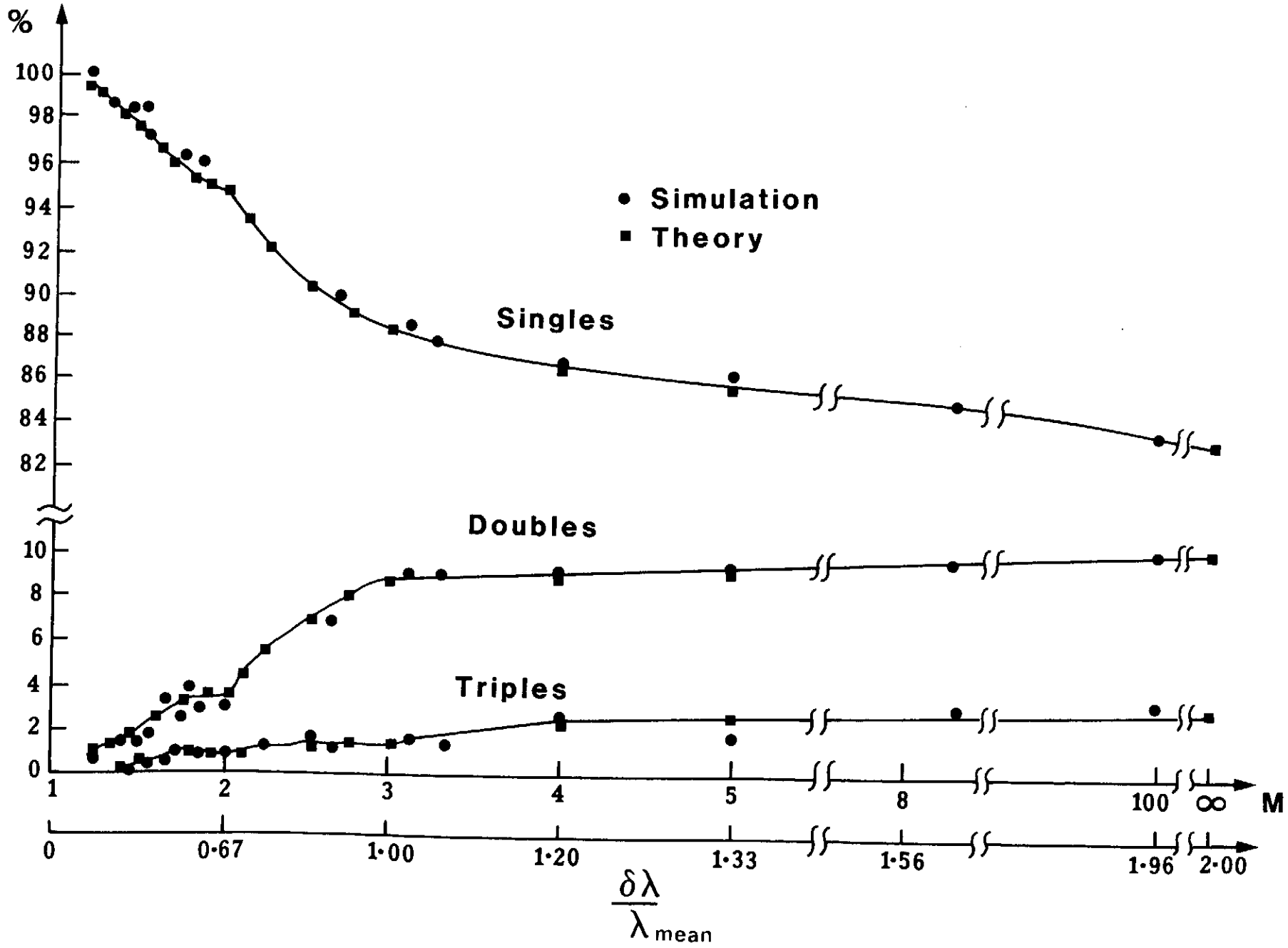
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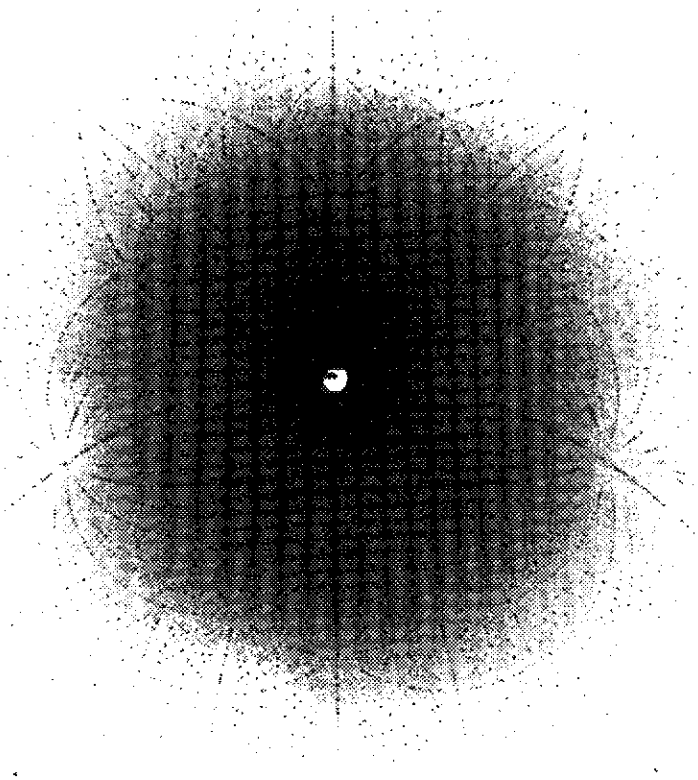
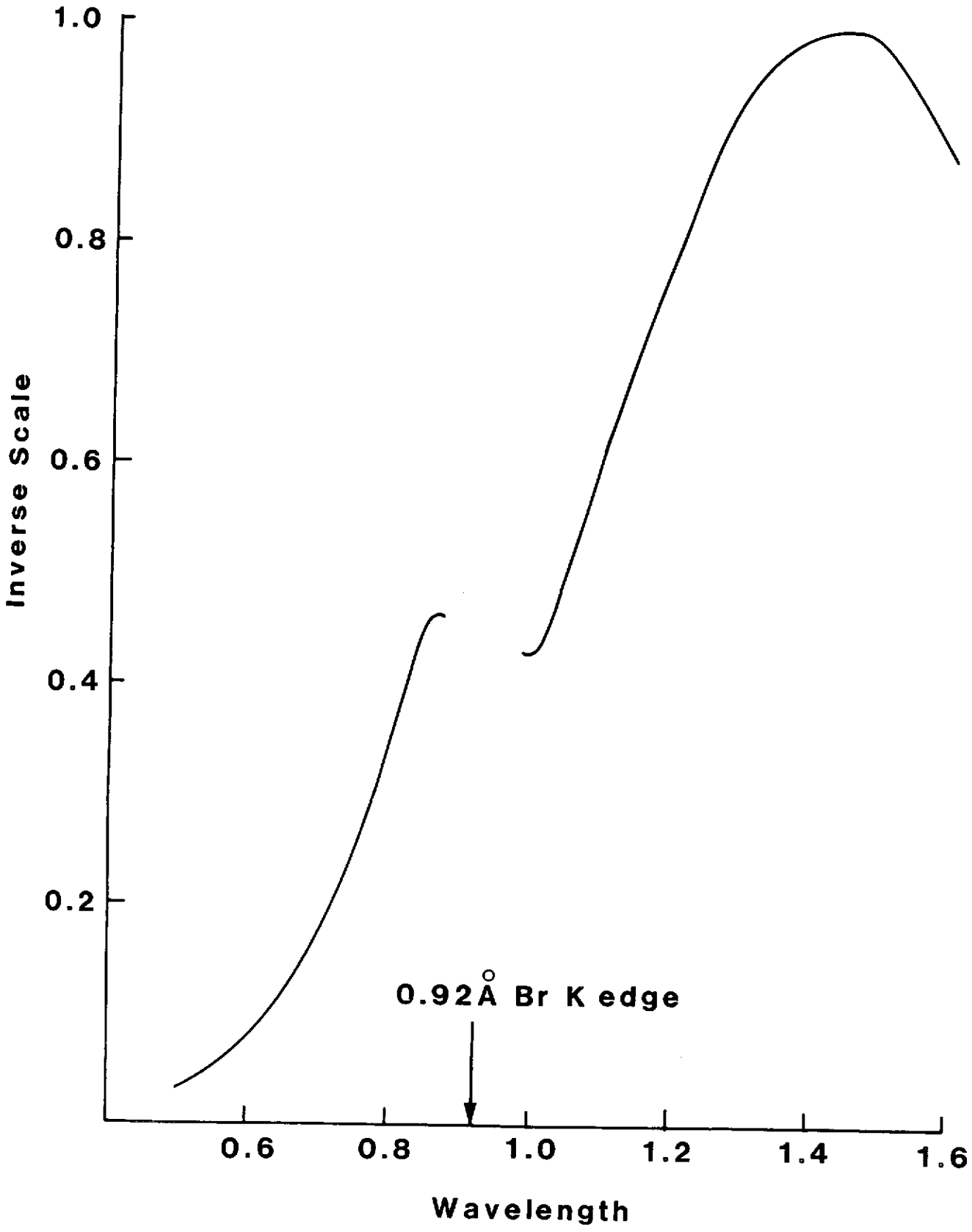
Figure 2

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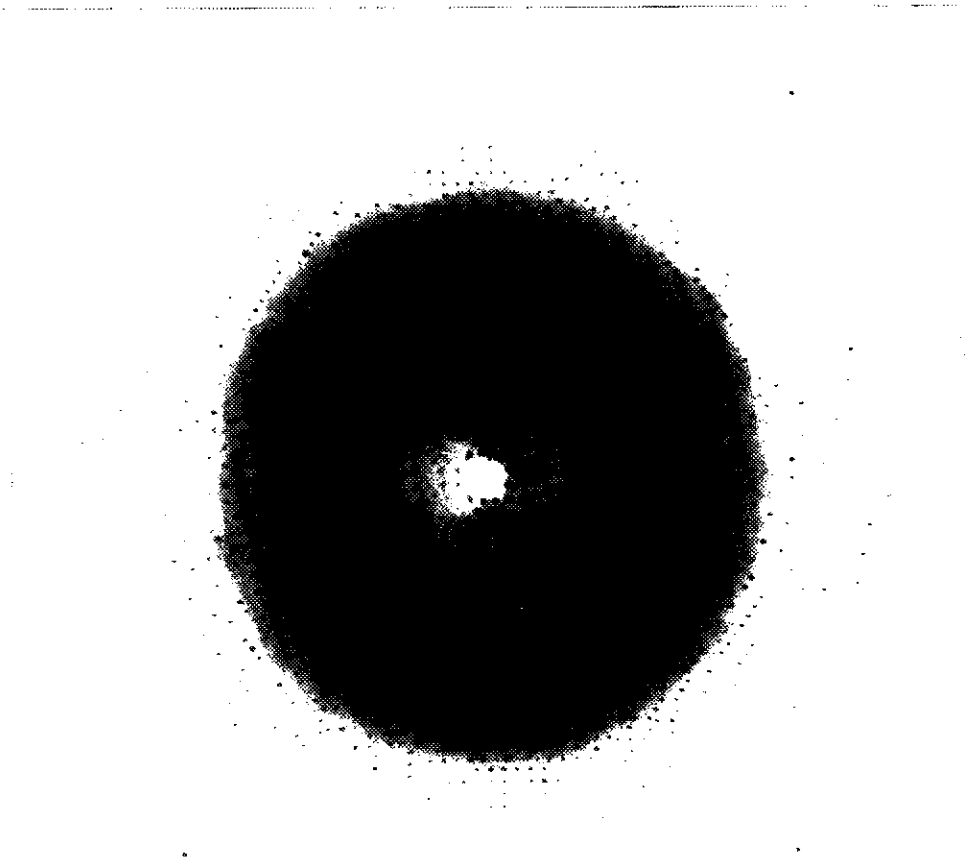
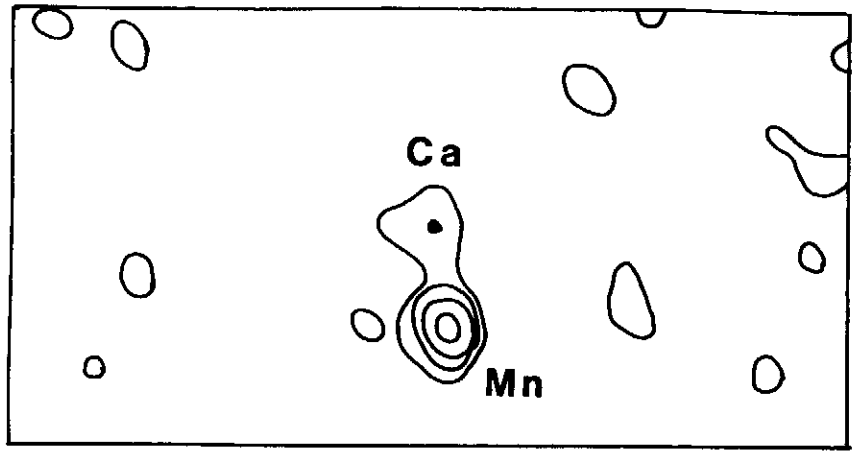
Wavelength

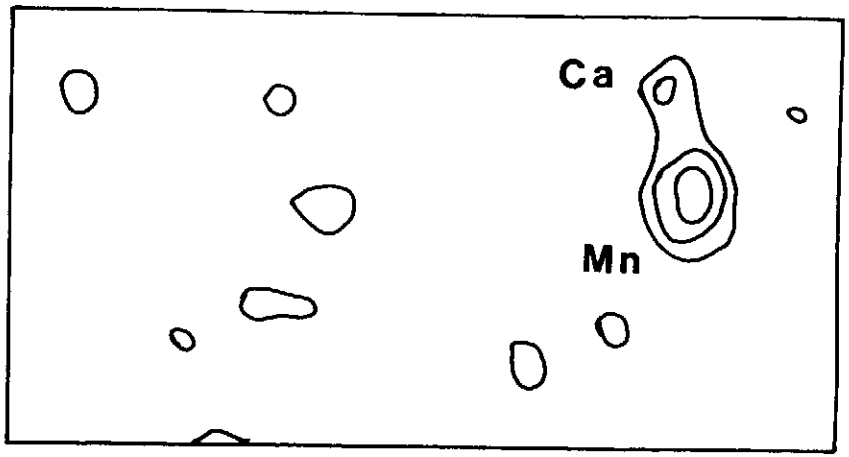
Figure 4



PEA LECTIN METAL COFACTOR SITES.

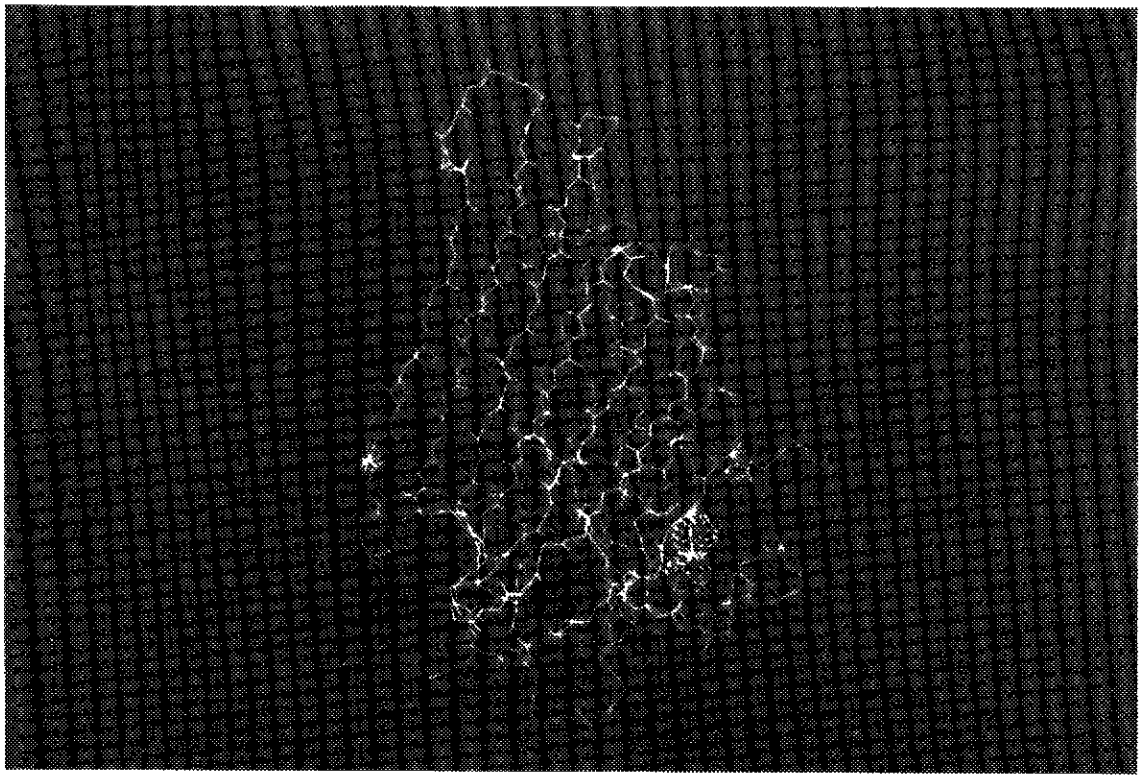


$\lambda = 1.860\text{Å}$ Monomer A



$\lambda = 1.860\text{Å}$ Monomer B

Figure 6



Comparison of three-dimensional protein structures in solution and in single crystals.

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The introduction of nuclear magnetic resonance (NMR) as a second method for protein structure determination (1) besides the well established X-ray diffraction technique with single crystals, is of fundamental interest, since it provides data that are in many ways complementary to those obtained from X-ray crystallography. Its use thus promises to widen our view of protein molecules with regard to a better grasp of the relations between structure and function, which provide the basis for research in protein design and protein engineering. The complementarity of the two methods results from the facts that the time scales of the two types of measurements are widely different, and that in contrast to the need of single crystals for diffraction studies the NMR measurements use proteins in solution or other noncrystalline states:

(i) Since protein structure determinations by NMR or by X-ray diffraction can be performed completely independently, meaningful comparisons of corresponding structures in single crystals and in non-crystalline states can be obtained. This is highly relevant, since the solution conditions for NMR studies can often be chosen so as to coincide closely with the natural, physiological environment of the protein. In such comparisons so far, both major conformational rearrangements between the two states (2,3) as well as extensive similarities between crystal and solution structures (4-6) have been observed. The α-amylase inhibitor Tendamistat (Fig.1) is one of the proteins for which the global molecular architecture is the same in crystals and in solution (4). A comparison of the refined structures in the two states, however, revealed numerous subtle differences. These are significant both with

respect to the functional properties of the protein, and with respect to the different information than can be obtained by the two methods for structure determination.

(ii) Since NMR can be applied to molecules for which no single crystals are available, new structures can be obtained that are not available from X-ray studies (7,8). Conversely, if the size of the protein exceeds a molecular weight of 20'000 to 30'000, or if it is not possible to prepare relatively concentrated (one to several mM), stable solutions, X-ray diffraction remains the only avenue to the molecular structure, provided that suitable single crystals are available.

(iii) For a characterization of the internal dynamics of proteins, NMR provides direct, quantitative measurements of the frequencies of certain high activation energy motional processes, and at least semiquantitative information on additional high frequency processes (1). The corresponding information from X-ray structure determinations commonly consists of an outline of the conformation space covered by the combination of static disorder and high frequency structural fluctuations.

(iv) A complementation of crystal structure data may result from the fact that the solution conditions for NMR studies (e.g., pH, temperature, ionic strength, buffers) can usually be varied over a wide range. This opens new avenues for comparative studies of proteins under native and denaturing solution conditions, for gaining novel insights into the problem of protein folding, and for investigations of intermolecular interactions with other solute molecules.

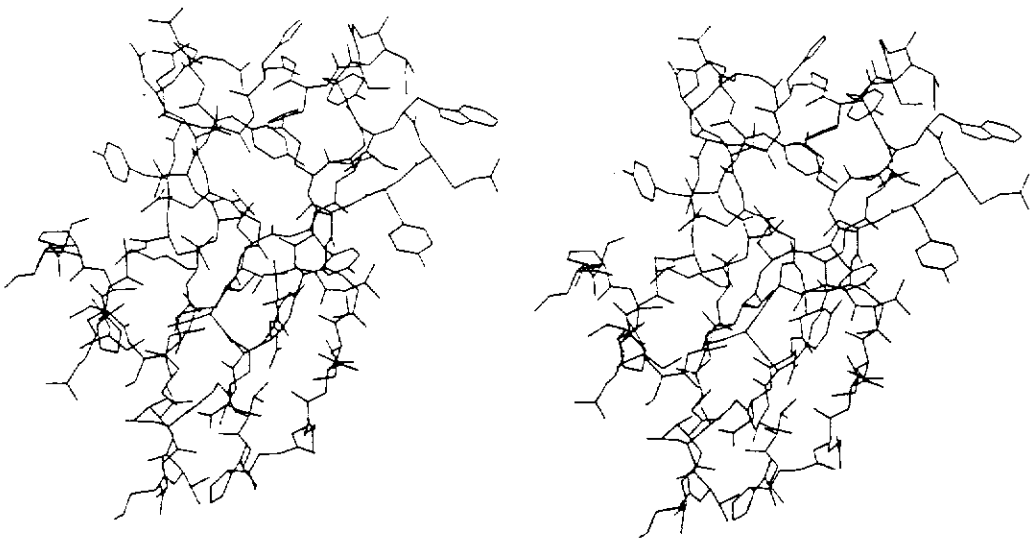

Fig. 1

Stereo view of the three-dimensional structure of the α-amylase inhibitor Tendamistat determined from NMR measurements in aqueous solution. All bonds connecting heavy atoms are shown for the residues 5-73.

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NMR Studies of DNA Structural and Dynamic Properties

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One of our long range goals is to understand how the biological function of DNA is related to its physical properties (conformation, conformational fluctuations, hydration, ion binding, etc.). On a more practical level, we would like to understand how the physical properties are dictated by the sequence of the DNA. A variety of different experimental techniques have been used to probe different aspects of DNA properties. In the work to be discussed here, we have used ^2H NMR to examine (i) the conformational heterogeneity and base motions in oriented DNA fibers, (ii) the effect of hydration on these DNA properties, and (iii) the properties of DNA liquid crystals.

Conformational Heterogeneity and Base Librations: Many key experiments have been on solid samples of DNA (crystals, fibers, films). X-ray diffraction studies on DNA fibers [1] and more recently on crystals have provided detailed information about DNA structures [2-5], but crystallographic data on B-form DNA oligonucleotides are limited [6-8]. There is also a problem in relating DNA structures in the solid and solution state since A-form DNA crystals have been obtained from molecules that are undoubtedly B-form in solution [4,9]. The amplitudes of bases motion in DNA are of interest because if they are large, they diminish the importance of local DNA structure on important events such as protein recognition of specific DNA sequences.

We have used solid-state deuterium NMR to examine the tilt angle and motional amplitudes of the purine bases in oriented films of DNA as a function of hydration level and temperature. For these studies, protons at the 8-position of guanine and adenine were exchanged with deuterons [10,11], and the wet-spinning method [12,13] was then used to obtain films of uniaxially oriented DNA. The example shown in Fig. 1 is for a DNA sample that has been hydrated at 66% RH, corresponding to a water content, w, of 7.7 H_2O/nucleotide. The best simulation of these spectra is obtained when a Gaussian distribution of $9° \pm 1°$ about an average base tilt of 0° is used. These results are consistent with early x-ray diffraction studies on B-DNA fibers that indicated that the average base tilt is 0° ($\langle\theta\rangle = 90°$) [14] and they agree with values of the tilt angles obtained by Fratini et al. (1982) for a crystal of a brominated DNA dodecamer in the B-form. To account for the observed spectral splitting (137 kHz in Fig. 1), an amplitude for the tilt motion, of 13° and torsional libration $10\pm1°$ is required. These are fairly large amplitudes of motion for DNA that is relatively dry.

Effects of Hydration on DNA: The above results refer to a sample containing 7.7 H_2O/nucleotide. At low hydration levels, a slightly increased distribution width is observed. The motional amplitudes are hydration dependent, with the tilting motion ranging from 4° for the driest, up to 15° for the wettest sample, and slightly larger amplitudes are observed for the twisting motion. At T = -60°C, the tilt motion, but not the twist motion, is suppressed with little increase in base disorder. With increased temperature and hydration, the bases execute an increased librational motion with a slightly larger twist than tilt amplitude. If we extrapolate to high levels of hydration, tilt and twist amplitudes greater than ±20° are expected.

Na-DNA: The Na salt of solid DNA is known to undergo a transition from the A-conformation at low relative humidities (RH), to the B-conformation at higher humidities. The exact transition point (86-96% RH) depends on sample preparation techniques and excess salt

concentration [15]. Since the x-ray diffraction pattern of a Na-DNA film at 75% RH showed the typical A-form reflections, it was surprising to find that the ^{2}H NMR spectrum of a folded film, shown in Fig. 2 (solid line), contains a large spectral contribution from B-form DNA (generating the large "horns" at ±68 kHz). To explain this spectrum, lineshapes of both the A and B form were calculated, and then added together with the proper weighting factor. The experimental spectrum presented in Fig. 2 (solid line) can be simulated (Fig. 2, broken line) by assuming that the sample contains 57% B-form DNA and 43% A-form DNA. The spectrum of A-DNA is very sensitive to base tilt angle, which can be determined accurately to be 23°±1°. This is slightly larger than the value of ~20°, found from x-ray diffraction of A-DNA fibers [1], and significantly larger than the value of ~12° observed for the purine bases in the A-form of a DNA oligonucleotide [16]. The combined distribution width of tilt angles and helix axes orientations in A-DNA at 75% RH is only~4°, compared with ~2.6° found by x-ray diffraction of an A-DNA dodecamer [16]. The observation that Na-DNA samples containing 1% excess salt *can* have up to 50-60% B-DNA at 75% RH suggests that caution be exercised when x-ray diffraction techniques are used to identify the conformational state of DNA in films. This is especially true in spectroscopic studies [17-21] of films where it is important to know the relative amounts of DNA in different conformational states.

DNA Liquid Crystals: It has been long recognized that concentrated DNA solutions can spontaneously organize to form cholesteric type liquid crystals, and DNA in certain organisms (bacteria, dinoflagellates [22-24]) appears to form cholesteric structures. Short fragments of DNA (100-200 base pairs in length) also form cholesteric liquid crystals [25-27] and in our work we demonstrated that these liquid crystals are magnetically ordered with the DNA axes perpendicular to the applied field (cholesteric pitch axis parallel to the magnetic field) (see Fig. 3A). In the present work, we have studied the ^{2}H spectra of DNA liquid crystals, prepared using DNA deuterated at the purine 8-position [28,29]. These studies provide information about certain structural and dynamical aspects of the DNA molecules in the liquid crystalline state, and they explain why we are able to observe spectra from moderately hydrated DNA films and liquid crystals whereas previous attempts to obtain spectra of deuterated DNA in a heavily hydrated solid state [28,30], or in solution failed [31].

The ^{2}H NMR spectrum obtained from a DNA sample with concentration 0.25 g/ml is shown in Fig. 3B. The spectrum has a splitting between the resonances, $\Delta\nu$, of ~42 kHz, and since T_{2e}~60 μs, the peaks are homogeneously broadened by at least ~5 kHz. Upon dilution of the sample by two fold, the DNA forms an isotropic solution and we were unable to observe any resonances from this diluted DNA sample, and this is consistent with earlier failures to obtain spectra from isotropic solutions of deuterated RNA duplexes [31]. To account for the observation of an NMR signal from DNA in the heavily hydrated liquid crystalline sample, we propose that the DNA molecules spin rapidly about their own axis so that the spectrum is cylindrically averaged, and the magnetic field creates a long-range order in the liquid crystal that prevents end-over-end tumbling of the DNA, while still allowing restricted angular fluctuations in the DNA orientation [32]. The observed spectra can be simulated assuming an average base tilt of 0°, but very substantial base librations in combination with fluctuations of the DNA axes about their mean position (combined effect ~37°). Note that because the C-D vector is oriented approximately along the long axis of the base, the measurements are relatively insensitive to base roll [30]. Therefore, while the base *tilt* is close to 0°, the base *inclination* could be different if the base pairs are propellered or if there is significant base roll. It will now be interesting to see how DNA length and other experimental parameters affect the properties of the DNA liquid crystals.

Acknowledgments

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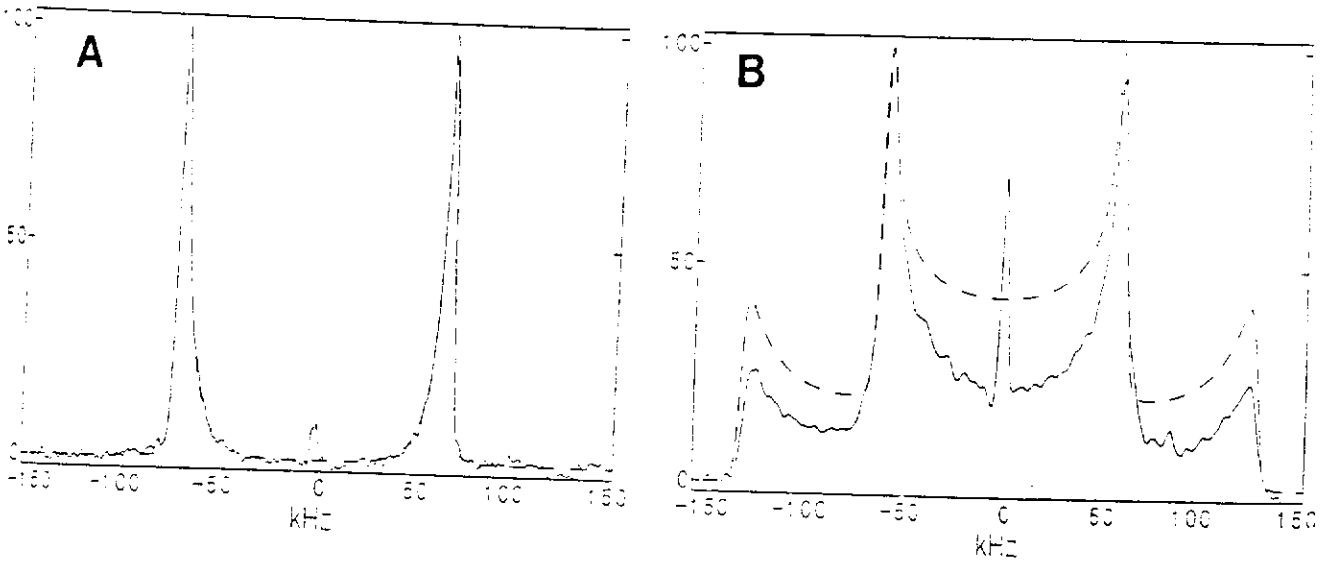

Fig. 1. Spectra of deuterium labeled Li-DNA at T = 25°C and w = 7.7 H_2O/nucleotide along with simulated spectra (broken lines): $\langle\theta\rangle = 90°$, $\sigma_{\langle\theta\rangle} = 0°$, $(e^2qQ/h) = 181$ kHz, $\eta = 0.06$, $\sigma_\beta = 9°$, $\theta_0 = 10°$ and $\phi_0 = 12°$. (A) Helix axis parallel to magnetic field. (B) Helix axis perpendicular to magnetic field.

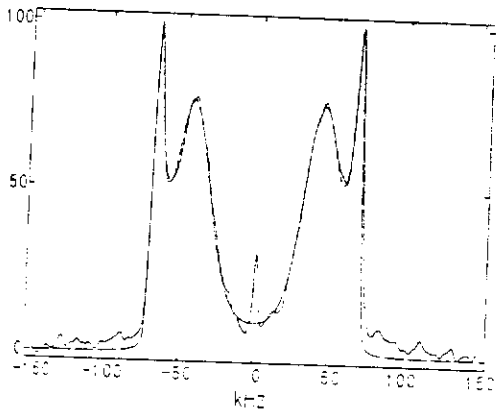

Fig. 2. Oriented film of Na-DNA, containing 1% excess NaCl, at 75% RH. Experimental ^2H NMR spectrum at $\beta=0°$ (——). Calculated spectrum (–·–).

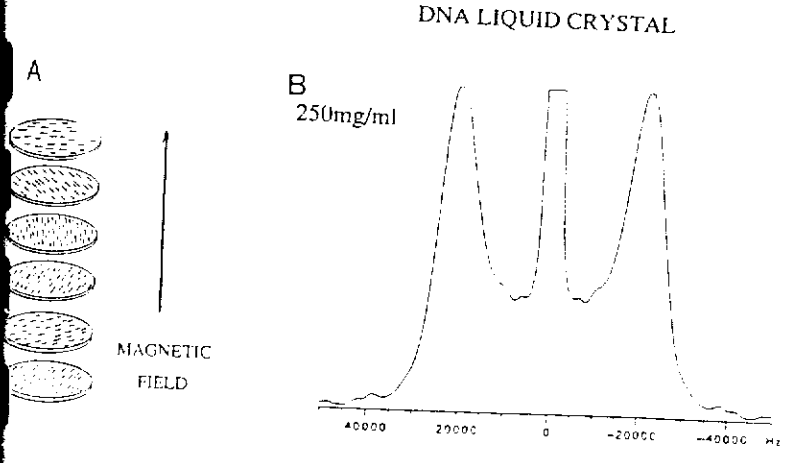

Fig. 3. (A) Idealized representation of a cholesteric liquid crystal showing the orientation of the DNA helix axes with respect to a magnetic field. (B) Deuterium NMR spectrum of ^2H labeled DNA at a concentration of 0.25 g/ml. This spectrum was obtained by subtracting out the residual HOD signal.

ELECTROSTATIC ENHANCEMENT OF DIFFUSION RATES TO THE ACTIVE SITES OF ENZYMES AND TO ION CHANNELS

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The transport of ions across membranes is a process of central importance in biology. The phospholipid bilayer poses a large barrier to the transport of charged species and most ions can only traverse this permeability barrier with the help of either carrier molecules or channels. Many channels allow ions to pass with diffusion rates close to those observed for the free ion in solution. Such channels are frequently highly selective and can efficiently discriminate betwen ions as similar as Na^+ and K^+. The properties of high selectivity and high permeability are somewhat contradictory since the former would appear to require a narrow channel while the latter would appear to require a wide channel. The problem can however be resolved if diffusion rates to the channel mouth are significantly larger than expected based on free diffusion. In this report I will discuss how enhanced rates of diffusion to small target areas can be achieved.

A suggestion that has appeared in the literature a number of times is that electrostatic potentials whose source is near the channel mouth enhance diffusion rates. The problem of translating this simple statement into a quantitative description of an enhanced diffusion process is quite complex since it requires a knowledge of the structure of the channel and of its charge distribution, the availability of methods to describe the electric fields produced by irregular bodies and the ability to describe, quantitatively, the diffusion process to such bodies. Unfortunately, except for the photosynthetic reaction center, the structure of membrane proteins have not been determined to atomic resolution. For this reason, we have chosen to study electrostatically enhanced diffusion to globular proteins whose structures are known.

In this report I will describe recent work we have carried out to described the electrostatic potentials of proteins and to calculate diffusion rates to irregular bodies. The electrostatic potentials are obtained by solving the Poisson-Boltzmann equation using numerical methods. The protein is treated as a low

dielectric cavity containing charges whose locations are defined by the coordinates of the relevant atoms in the x-ray structure. The solvent is described as a high dielectric continuum containing a simple electrolyte.

Diffusion rates are calculated from an extension of a Monte-Carlo algorithm for solving the three- dimensional Smoluchowski equation. The algorithm uses extensive table lookups to describe the molecular shape and force field in order to reduce computation time. This allows the algorithm to be vectorized.

These methods are applied to study the diffusion of the superoxide anion, O_2^-, to the protein Cu,Zn superoxide dismutase (SOD). It is found that the shape of the active site channel of SOD focusses the electric field of the enzyme into solution leading to a large capture cross-section for the anion. The diffusion rates is found to be enhanced significantly by the presence of the electric field and provides a basis for the interpretation of seemingly paradoxical experimental results. The implications of this study for membrane transport as well as for other biologically relevant diffusion processes will be discussed.

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MERCREDI 6 JUILLET 1988

9 h - 18 h

BIOLOGIE ET MATHEMATIQUE DES RESEAUX NERVEUX

BIOLOGY AND MATHEMATICS OF NEURAL NETWORKS

BIOLOGY AND MATHEMATICS OF NEURAL NETWORKS

PROPERTIES OF NEURAL NETWORKS

JOHN C. ECCLES

Chairman's Address

Almost 40 years ago (Eccles, 1951) I was diagramming neuronal networks that I hoped might give some insight into the complexity of cortical responses. They were built on the erroneous assumption of a network of excitatory synaptic connectivities. If each neurone was directly connected to two or to three others and so on sequentially, the neuronal patterns could be drawn in 2 and 3 dimensional geometry respectively. Calculations were made for the large progressive increases in neuronal involvement within 20 msec for connections to 4 or 5 neurones using 4 and 5 dimensional geometry and the simplest initial conditions.

This work was given up in the light of the magnificent microstructural studies on the neocortex with the complemental functional studies. Of particular significance has been the discovery of the interacting inhibitory neuronal systems of the neocortex as described so authoritatively by Szentágothai (1978,1983) for example. Unfortunately all of this fundamental work on neocortical structure and function is not even mentioned in recent accounts of the cerebral neocortex. (Changeux,1985; Edelman, 1987). Nor is it utilized in all of the very sophisticated neural modelling in the mathematical network theories. I will therefore make brief reference to the modern knowledge of the association neocortex, which accounts for about 95% of the human neocortex.

Radiotracer investigations (Goldman and Nauta, 1977) have revealed a modular structure of connectivities, as is illustrated in Fig.1. Each module is a vertical assemblage of neurones across the cortex and about 300 μm across. This assemblage tends to behave in a global manner in receiving from and in projecting to other modules of the same or other hemisphere (Fig.1). There are some thousands of neurones in such a module about 60% being the excitatory pyramidal cells that communicate to other cortical modules or that project out of the cortex. (Szentágothai, 1978).

As indicated in Fig.2 (Szentágothai, 1983) there is a wide variety of neurones within a module, both excitatory and inhibitory (shown in black). Their complex interconnections provide tha basis for the inner dynamic life of a module. Diagrammatic simplification is achieved by drawing the inhibitory neurones in black and to the left of the module, as explained in the legend. The identification of all neurone types in Fig.2 is shown in Fig.3. Simplicity is achieved by drawing only one of each type of neurone, labelled with a geometrical symbol that allows identification by the key to the right.

As shown in Fig.2 the output from a module is exclusively by pyramidal cells, while there are two main inputs, the specific afferents from the thalamus that terminate in excitatory synapses in lamina 4, and the cortico-cortical afferents from other modules (Fig.1) that dominate the synaptic input to the module at most laminae, but not lamina 4. Furthermore these cortico-cortical afferents bifurcate in lamina 1 to project for several millimeters horizontally to many adjacent modules, as can be seen in Fig.4 when they traverse 3 modules making excitatory synapses on the apical dendrites of pyramidal cells. It has been estimated that the apical dendrites of each pyramidal cell receive 1000 to 3000 such synapses (Szentágothai,1978), which is a potentially powerful input.

It will be noted in Fig.4 that the apical dendrites of 3 pyramidal cells are partly indicated in close apposition. It has now been shown that the apical dendrites of about 20 pyramidal cells are thus closely approximated in a bundle and are spaced at some distance from the next bundle, so that within the module there appear to be many mini-columns - about 50. It is conjectured that this bundling into minicolumns has important functional significance for the mind-brain problem (Eccles,1986).

Reference also should be made to the axon collaterals of the pyramidal cells (Fig.5) which are widely distributed to adjacent columns, but which are most intensely distributed to the module of origin. They would provide an effective positive feed-back to the pyramidal cells of an activated module.

It should now be sufficiently evident that no meaningful modelling of the cerebral cortex can be achieved if all these complexities are ignored. The actual situation is operationally far from the simplified network theories that have been developed in attempts to model the cerebral cortex. However, it should be possible to develop models that

incorporate some of the principal features of the cerebral cortex, for example the two distinctive excitatory inputs and the inhibitory control. Szentágothai (1978) estimates that each pyramidal cell projects significantly to about 50 pyramidal cells in other modules (Fig.1) and receives from a like number. So eventually the modelling of the cerebral cortex would involve 50 - dimensional geometry!

I apologize for introducing this chilling atmosphere of realism into the cozy world of elegant model building.

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LEGENDS

Fig.1: A. The general principle of cortico-cortical connectivity is shown diagrammatically in a nonconvoluted brain. The connections are established in highly specific patterns between vertical columns of 200-300 µm diameter in both hemispheres. Ipsilateral connections are derived mainly from cells located in layer III (cells shown at left in outlines), while contralateral connections (cells shown in full black) derive from all layers II-VI. The diagram does not try to show the convergence from afferents originating from different parts of the cortex to the same columns. B. Golgi-stained branching of a single cortico-cortical afferent, oriented in relationship to the module with a single afferent in A, but at several times higher magnification. It illustrates the profuse branching in all laminae.Bar=100 µm (Szentágothai, 1978)

Fig.2: Internal neurone connectivity in a cortico-cortical column or module, the vertical cylindrical space of about 300 µm in the centre. The module is sharing part of its space with two flat discs in lamina IV in which specific afferents (Spec.Aff.) arborize. The cortico-cortical afferents (indicated at bottom) terminate all over the cortico-cortical module, though with different densities of terminals. In lamina I, the tangential spread of the cortico-cortical fibres extends far beyond the module. The selection of pyramidal cells for output is envisaged in the right half of the diagram over excitatory interneurons (Ss-spiny stellate or over disinhibitory interneurons, the CDB (cellule à double bouquet of Ramón y Cajal), which is an inhibitory interneurone that acts specifically upon inhibitory interneurones which are shown in full black. The left side of the diagram explains the action of inhibitory interneurones as some kind of 'filter' keeping out of action some of the pyramidal cells (punctate shading). Interneurones that can be defined as inhibitory with a considerable amount of confidence are indicated in full black, the basket cells, BC, in the deeper laminae, the SBC (small basket cells) in lamina II, the axonal tuft cells, ATC, and a very specific axo-axonic cell, AAC, acting upon the initial segments of pyramidal cell axons (Szentagothai,1983).

Fig.3: Simplified diagram of modular design in the association neocortex, the laminae being indicated to the left. In A there are shown the excitatory cells with their excitation by the cortico-cortical (CC) and thalamo-cortical (TC) inputs. In B there are the synaptic connectivities for inhibitory cells. The cell identifications are by symbols except

for the pyramidal cell in the centre of each diagram. The symbols to the lower right are arranged in the same depth order as the cells in the diagram: ATC, axonal tuft cell; CDB, cellule à double bouquet; SBC, small basket cell; LBC, large basket cell; Ng, neurogliform cell; Sst, spiny stellate cell; AAC, axo-axonic cell; MC, Martinotti cell. CS indicates cartridge synapse. All excitatory cells and synapses are in outline. All inhibitory cells and synapses are in solid black.

Fig.4: Simplified diagram of connectivites in the neocortex that is constructed in order to show pathways and synapses.

. The diagram shows three modules, A,B,C. In laminae I and II there are horizontal fibres arising as bifurcating axons of commissural (COM) and association (ASS) fibres and also of Martinotti axons (MA) from module C. The horizontal fibres make synapses with the apical dendrites of the stellate pyramidal cell in module C and of pyramidal cells in modules A and B. Deeper there is shown a spiny stellate cell (Sst) with axon, AX, making cartridge synapses with the shafts of apical dendrites of pyramidal cells (Py). Due to conjunction hypertrophy the association fibre from module C has enlarged synapses on the apical dendrites of the pyramidal cell in module A.

Fig.5: Diagram illustrating long range intracortical connections. Two Martinotti cells shown in lamina VI with ascending axons bifurcating in lamina I. Pyramidal cell in lamina II has widely distributed axon collaterals. One cell in lamina IV has a bifurcating horizontal axon. Arrow shows cortico-cortical afferent (Szentágothai, 1978).

FIGURE 1

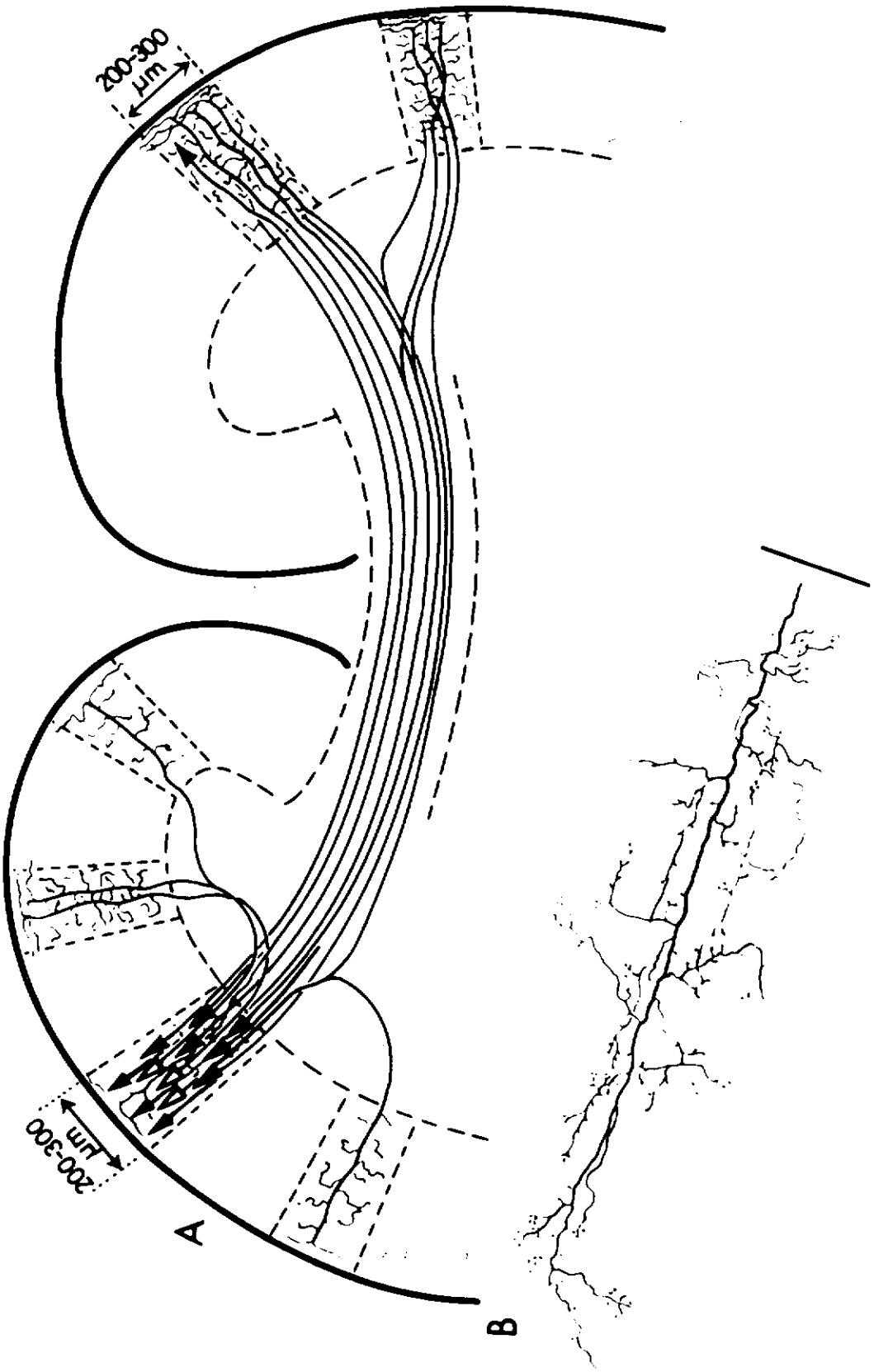


FIGURE 2

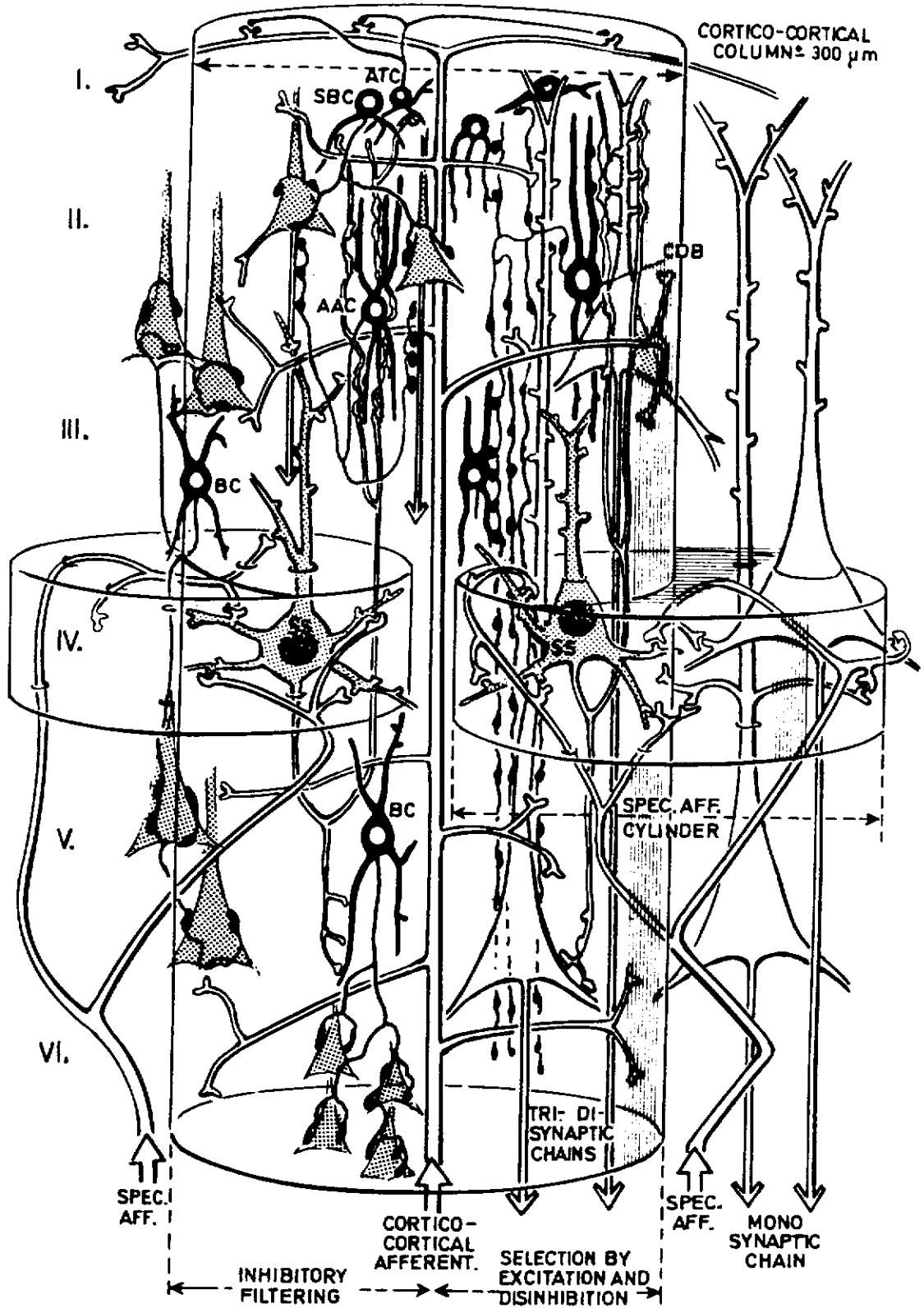

CORTICO-CORTICAL
COLUMN ≈ 300 μm
I.
II.
III.
IV.
V.
VI.
ATC
SBC
AAC
CDB
BC
SS
BC
SPEC. AFF.
CYLINDER
TRI- DI-
SYNAPTIC
CHAINS
SPEC.
AFF.
CORTICO-
CORTICAL
AFFERENT.
SPEC.
AFF.
MONO
SYNAPTIC
CHAIN
INHIBITORY
FILTERING
SELECTION BY
EXCITATION AND
DISINHIBITION

FIGURE 3

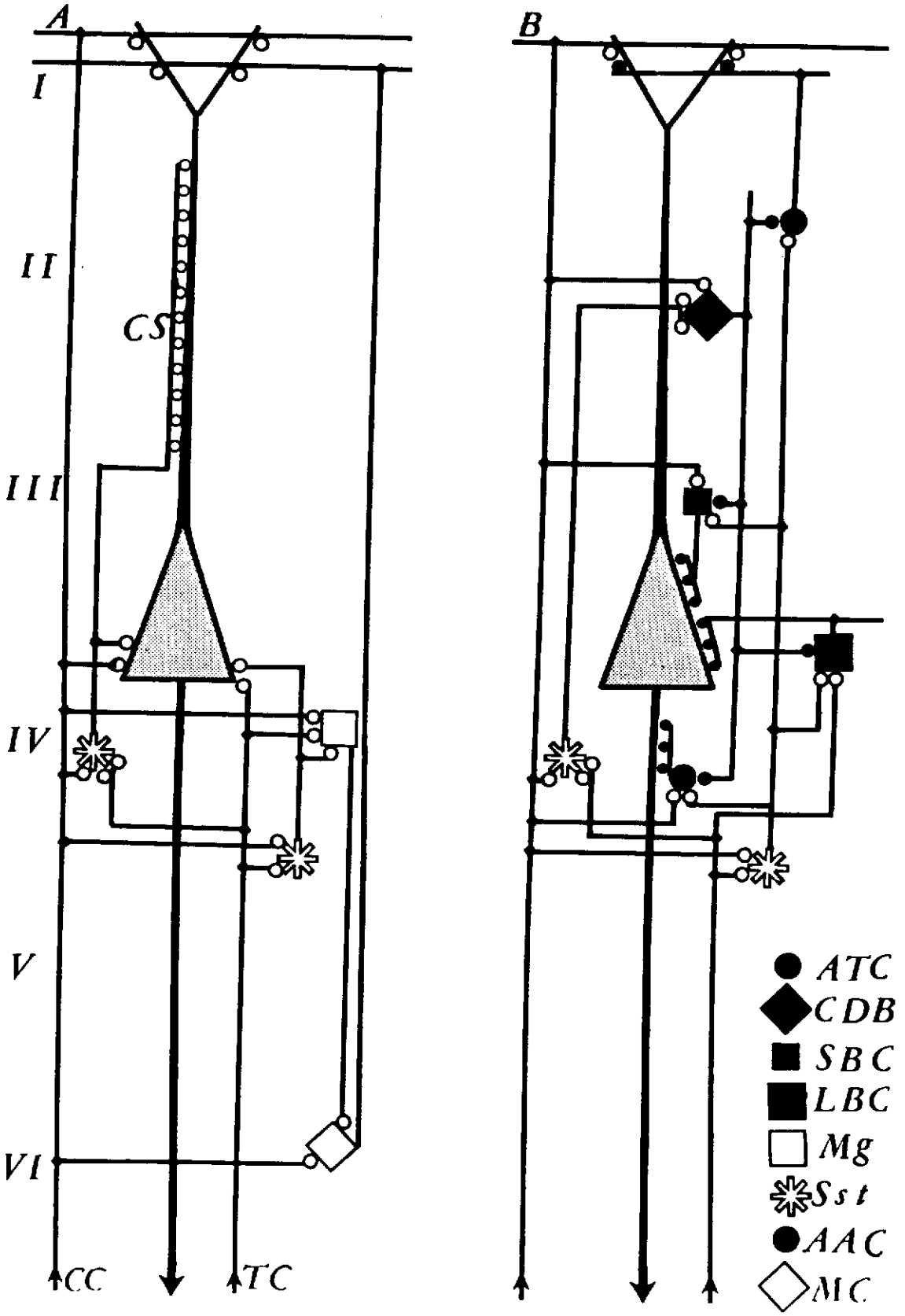


FIGURE 4

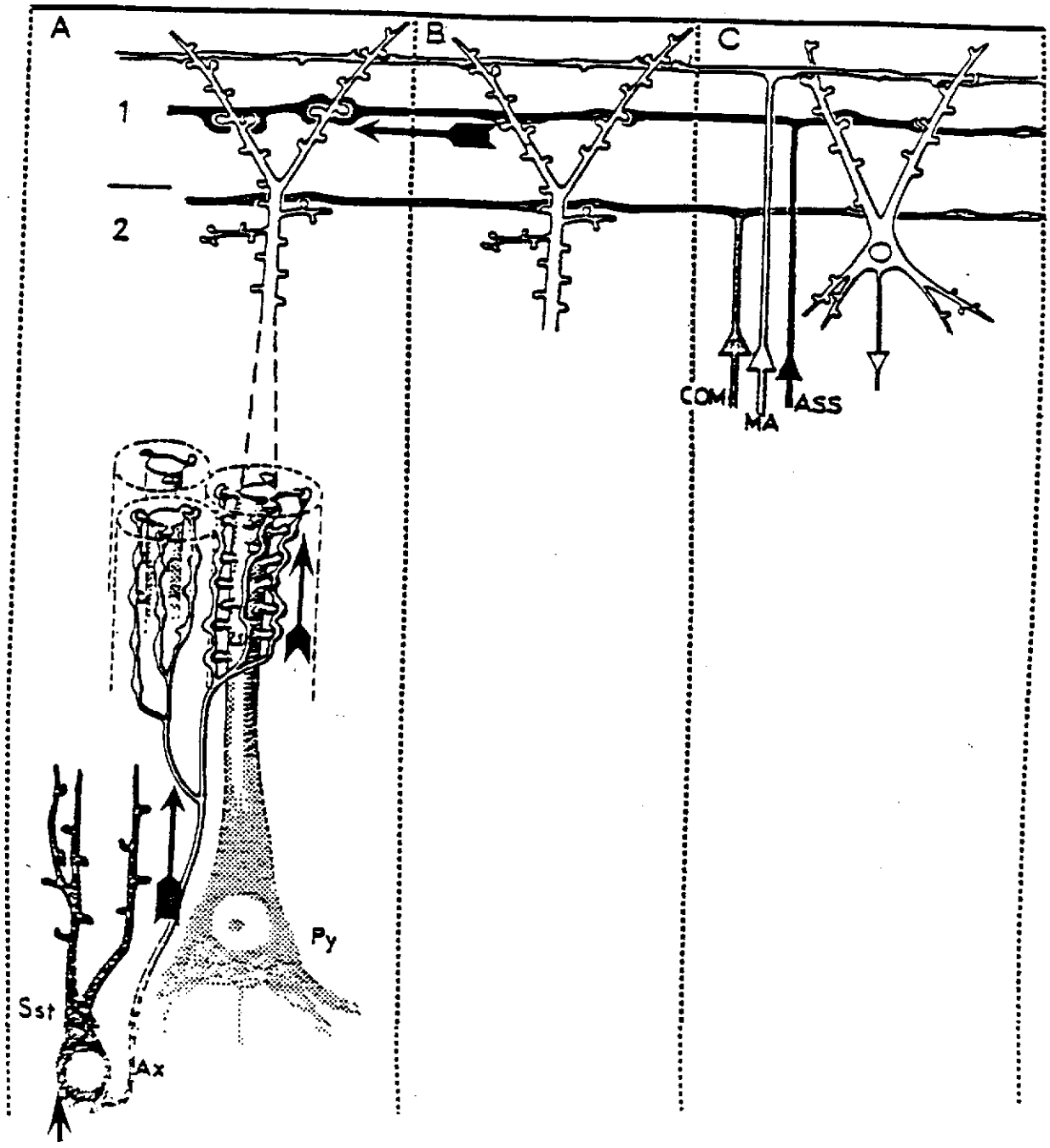
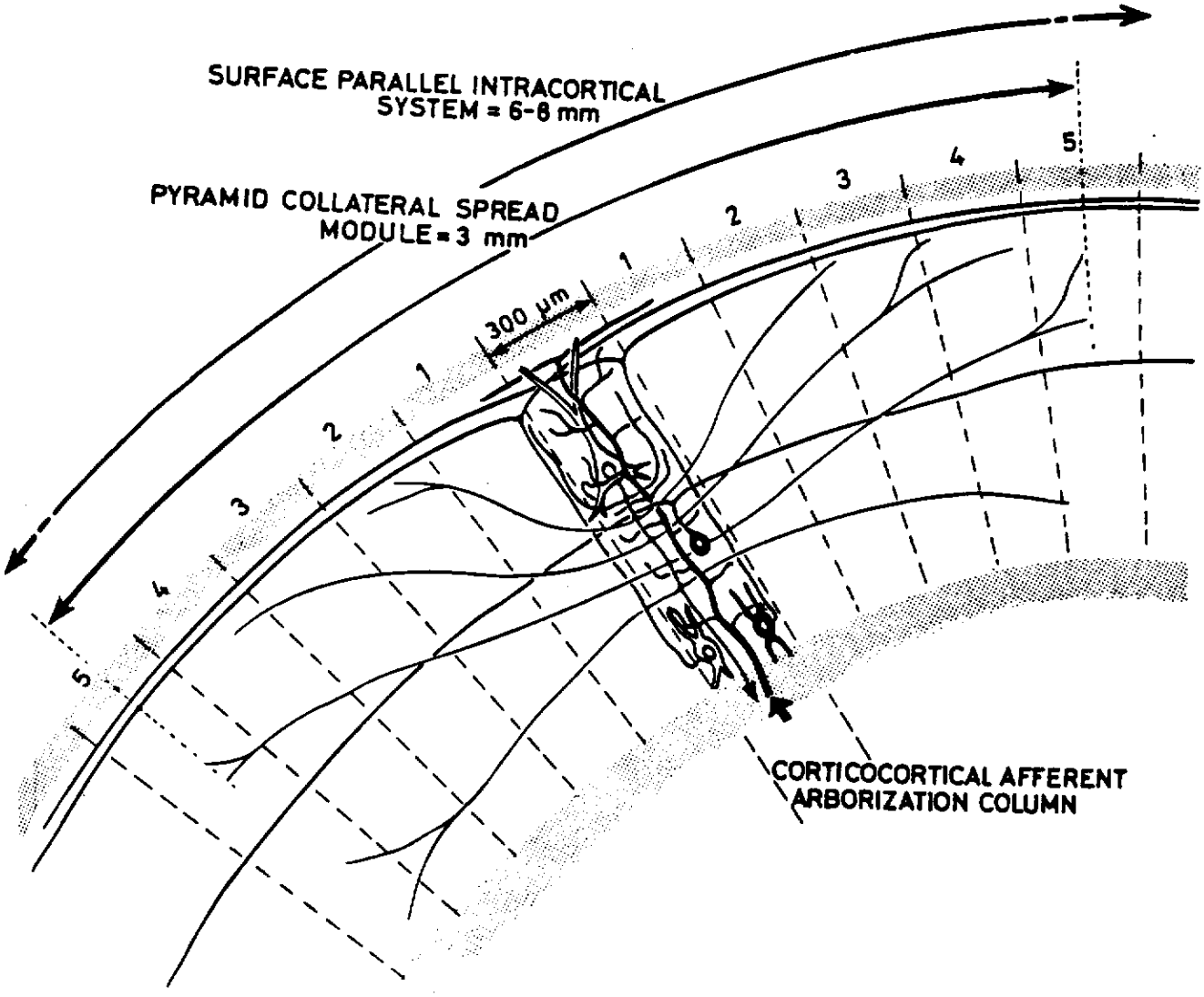


FIGURE 5


SURFACE PARALLEL INTRACORTICAL SYSTEM = 6-8 mm
PYRAMID COLLATERAL SPREAD MODULE = 3 mm
300 µm
1
2
3
4
5
CORTICOCORTICAL AFFERENT ARBORIZATION COLUMN

Mean-Field Theory of a Neural Network

Leon N Cooper

In recent years it has become increasingly fashionable to treat the brain as a set of neural networks: assemblies of neurons connected to one another by synaptic junctions that serve to transmit information and possibly to store memory. Since the contents of memory must depend to some extent on experience, neural networks and, in particular, the synapses between neurons cannot be completely determined genetically. This evident reasoning has led to much discussion about possible modification of synapses between neurons as the physiological basis of learning and memory storage. To properly function, neural network models require that vast arrays of synapses have the proper strengths. A basis problem becomes how these synapses adjust their weights so that the resulting neural network shows the desired properties of memory storage and cognitive behavior.

The problem can be divided into two parts. First, what type of modification is required so that in the course of actual experience the neural network arrives at the desired state? The answer to this question can be illuminated by mathematical analysis of the evolution of neural networks using various learning hypotheses. The second part of this problem is to find experimental justification for any proposed modification algorithm. A question of extraordinary interest is: What are the biological mechasisms that underlie the nervous system modification that results in learning, memory storage, and evntually cognitive behavior?

One experimental model that appears to be well-suited for the purpose of determining how neural networks modify is the cat visual cortex. The modification of visual cortical organization by sensory experience is recognized to be an important component of early postnatal development Although much modifiability disappears after the first few months of life, some of the underlying mechanisms are likely to be conserved in adulthood to provide a basis for learning and memory. We have approached the problem of experience-dependent synaptic modification by determining theoretically what is required of a mechanisms in order to account for the experimental observations in visual cortex. This process has led to the formulation of hypotheses, many of which are testable with currently available techniques.

A single cell theory for the development of selectivity and ocular dominance in visual cortex has been presented previously by Bienenstock, Cooper and Munro (BCM) This has been extended to a network applicable to layer 4 of visual cortex. In this paper we present a mean field approximation that captures in a fairly transparent manner the qualitative, and many of the quantitative, results of the network theory.

Having defined a mean field approximation that greatly simplifies the equations for the response and evolution of cortical cells, we have obtained a fundamental result: the stability and position of the fixed points in this network are related to the fixed points in the absence of mean field ($\alpha^{l(r)} = 0$) by

$$m_i^*(\alpha) = m_i^*(0) + \alpha\,,$$

where $m_i^*(\alpha)$ is a fixed point in the mean field α, while $m_i^*(0)$ is a fixed point of zero mean field.

Thus if $m_i^*(\alpha)$ is restricted to the first quadrant (positive values for all of its components due to the excitatory nature of LGN-cortical synapses), as long as α is large enough and non-specific (there is sufficient inhibition for all pattern inputs), $m_i(\alpha)$ can still reach all of the fixed points that would have been reached by $m_i(0)$ (not restricted to the first quadrant.) This means that if network inhibition is sufficient, the selective stable fixed points can be reached even though LGN-cortical synapses are excitatory. Once reached, the fixed points, $m_i^*(\alpha)$, have the same stability characteristics as the corresponding $m_i^*(0)$.

We find, consistent with previous theory and with experiment, that most learning can occur in the LGN-cortical synapses; inhibitory (cortico-cortical) synapses need not modify.

Some non-modifiable LGN-cortical synapses are required. It becomes interesting to ask whether these could be associated with some anatomical feature (e.g. might these be synapses into shafts rather than spines).

As in the zero mean field theory, zero cell output is an unstable fixed point. Thus in binocular deprivation the cell output could be on average above or below spontaneous activity (depending on the level of inhibition). Some 'non-visual' cells would reappear if excitation were enhanced or inhibition diminished.

In monocular deprivation the closed eye response goes to

$$c = (x - \alpha) \cdot d \longrightarrow 0$$

Therefore, LGN - cortical synapses do not go to zero. Rather

$$x \longrightarrow \alpha.$$

Thus if inhibition is suppressed one would expect some response from the closed eye. This is in agreement with experiment.

Various models for memory storage and retrieval have been suggested. These differ in several ways. One of the most important from the point of view of computational complexity as well as for realization in silicon is the degree of connectivity of each unit. What is suggested here is that much that is significant in at least one layer of visual cortex can be obtained in a primarily feed forward network of very simplified lateral connectivity. The original connectivity in which each of

the N neurons in this layer of cortex is connected to every other [N^2connectivity] can be replaced by a mean field network in which a neuron receives n LGN inputs and a single (mean field) input [(n+1) connectivity].

Magnetic Fields Associated with Neural Currents

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The human brain is organized as a hierarchy of processing sytems, which differ in their number of neurons, complexity of interconnections, and degree of spatial localization. These functions range from specialized single-cell responses and the direct communication between adjacent cells up through the perceptual, behavioral, and cognitive consequences of activity of extensive, distributed populations. Intense study has been underway in many laboratories during the past score of years to characterize single cell electrical properties in animal subjects by measurements with fine electrodes. This approach has been particularly successful for sensory cortex where neural response follows a controllable stimulus. More recently, interest has developed in multi-electrode explorations of the correlation between activities of cells within confined areas of cortex. The scale of investigation here ranges up to a hundred electrodes, in attempts to reveal the underlying interconnections and affects of the biochemical environment. Somewhat coarser, but with a broader view, are optical investigations of the pattern of fluorescence of an electric-field sensitive medium applied to the exposed surface of the brain. These patterns clearly delineate differing functional areas across the cortex. Even larger in scale, and with the whole brain of the human in view, is activity revealed with positron-electron annihilation tomography (PET), characterizing metabolic activity or oxygen uptake in centimeter-size volumes. Even finer temporal resolution is obtained by electrical measurements of the potential distribution across the scalp, or electroencephalogram (EEG). However EEG measurements as conventionally carried out are difficult to interpret in terms of the locations of underlying neural sources. I shall point out how the magnetic counterpart, or magnetoencephalogram (MEG), can be brought to bear successfully to localize neural activity in both cortex and subcortical areas of the human brain (Williamson et al., 1983). The interpretation of these data reveals functional architectures of the underlying neural circuitry, involving networks with more than 1,000 neurons. Neuromagnetism is proving a useful approach to both fundamental studies of brain function and certain evaluations of abnormal activity.

Both spontaneous and sensory-evoked magnetic signals from the brain have been investigated, but the greatest attention has been devoted to the latter. These are made possible by the extreme sensitivity of an invention that came from the laboratories of low-temperature physicists – the superconducting quantum interference device (SQUID). Neuromagnetic studies are done by placing SQUID magnetic field sensors near the scalp and recording the time-course of signals as the brain responds to a suitable sensory stimulus (Williamson and Kaufman, 1986). To minimize interference from time-varying magnetic fields of the environment, measurements can be carried out within a magnetically shielded room, or with detection coils of a suitable geometry in an unshielded environment. Signals of interest are very weak, typically 100 femtotesla or less ($< 10^{-13}$ T), a value that may be appreciated by comparison with the earth's much stronger steady field of about 70 microtesla (7×10^{-5} T). The theory of its origin was verified by *in vitro* studies of the magnetic field associated with the action potential of the crayfish axon (e.g., Roth and Wikswo, 1985). The neuromagnetic field as conventionally measured arises from intracellular currents flowing within active neurons. Moreover, since the human head is transparent to magnetic fields below a frequency of a few kilohertz, the observed field pattern of a confined neural

source can be interpreted by a simple procedure to reveal the source's location in three-dimensional space (Williamson and Kaufman, 1981). In this way sources have been identified in sensory areas, association areas, and hippocampal formation.

Here I shall take the example of auditory evoked fields, which have been clearly identified as lying in or near auditory cortex (Elberling et al., Farrell et al., 1980; Hari et al., 1980). Functional maps within auditory cortex have also been discovered (Romani, Williamson, and Kaufman, 1982a). In some cases these maps are similar to functional progressions observed by microelectrode studies of individual neurons in various species, but the neuromagnetic analysis indicates that the observed magnetic sources represent populations of about 10,000 neurons. Figure 1 gives an example of a tonotopic map describing such 'macroscopic' neural activity in auditory cortex. Magnetic resonance images of the subject indicate that all these sources lie within the Sylvian fissure directly above the ear canal, where auditory cortex is normally found. The tonotopic map can be accurately represented as a logarithmic sequence: the cumulative distance across the cortex from one region of activity to the next is proportional to the logarithm of the tone frequency. Another feature of the data also merits emphasis: Even though the observed fields depend on tone frequency, the strengths of the deduced sources do not (Romani, Williamson, Kaufman, and Brenner, 1982b). In other words, a common pattern of neural activation is produced by each tone, only the center of activity is shifted across cortex systematically with changes in tone frequency.

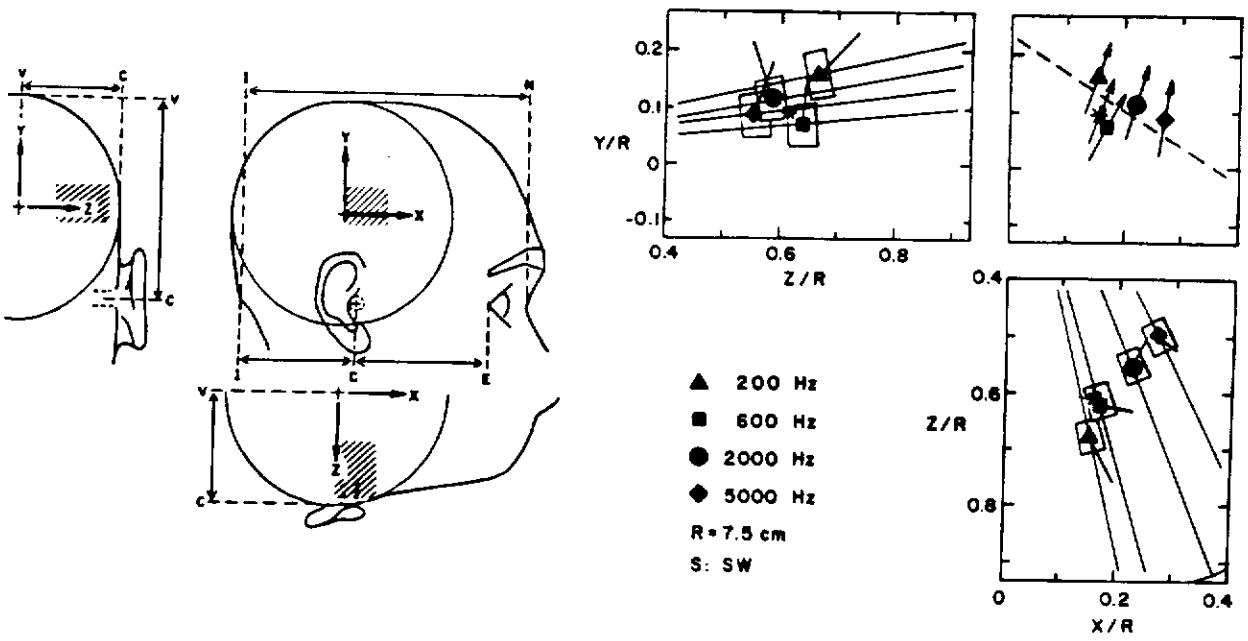

Figure 1. Locations of neural activity evoked by tones of the indicated four frequencies. The shaded rectangles in the cross sections of the head at the left are enlarged at the right to show locations of magnetic sources. Dimensions for the axes X, Y, Z at the right are expressed in units of the radius of the head: R = 7.5 cm. Outlined boxes indicate the uncertainty in neural source positions, and short lines indicate the orientations of the source currents. The dashed line in the upper right panel indicates the Sylvian fissure as prescribed by a standard anatomy text. Solid lines are radii from the best-fitting sphere employed to model the subject's head. (Adapted from Romani et al. 1982b)

Pantew et al. (1988) recently verified the existence of such a tonotopic map in a neuromagnetic study using a different form of auditory stimulus. Moreover, Hoke et al. (1988) have reported neuromagnetic evidence for an amplitopic sequence, whose locus across cortex lies approximately perpendicular to the frequency locus. Each 10 dB increase in stimulus intensity produces a shift of the neural activity by about 3 mm across cortex. I shall point out that these functional maps have an interesting relationship to psychophysical studies of perception, and the two measures taken together have important physiological implications for the architecture of the underlying neural circuits.

Neuromagnetic studies of higher functions of the brain have just begun. Of relevance to audition is an investigation of attentional affects on cortical activity. Curtis et al. (1988) found that when a person listens to one of two tunes presented simultaneously, cortical activity evoked by the attended tune is enhanced by a factor of two over activity evoked by the ignored tune. Attentional affects are seen all along the tonotopic map, indicating that activity over wide areas of cortex can be modulated by attention. By comparison, earlier brainstem activity is free from such influences.

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POSSIBLE MECHANISMS OF EXPERIENCE-DEPENDENT SYNAPSE MODIFICATION IN THE VISUAL CORTEX

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The cat visual cortex has proved to be a valuable experimental model for the purpose of determining how neural networks modify according to experience. Neurons in the primary visual cortex, area 17, of normal adult cats are sharply tuned for the orientation of an elongated slit of light and most are activated by stimulation of either eye. Both of these properties -- orientation selectivity and binocularity -- depend on the type of visual environment experienced during a critical period of early postnatal development (reviewed by Frégnac and Imbert, Physiol. Rev. 64: 325; 1984). For example, monocular deprivation (MD) during the critical period [extending from approximately 3 weeks to 3 months of age in the cat] has profound and reproducible effects on the functional connectivity of striate cortex. Brief periods of MD will result in a dramatic shift in the ocular dominance (OD) of cortical neurons such that most will be responsive exclusively to the open eye.

The consequences of binocular deprivation (BD) on visual cortex stand in striking contrast to those observed after MD. While 7 days of MD during the second postnatal month leave few neurons in striate cortex responsive to stimulation of the deprived eye, most cells remain responsive to visual stimulation through either eye after a comparable period of BD. Thus, it is not merely the absence of patterned activity in the deprived geniculocortical projection that causes the decrease in synaptic efficacy after MD.

Gunther Stent, in an influential 1973 paper (Proc. Natl. Acad Sci. USA 70: 997), suggested that the crucial difference between MD and BD is that only in the former case are cortical neurons active. According to this "learning rule", postsynaptic activation is necessary for all synaptic modifications and the sign of the change (+ or -) is dependent on the concurrent level of presynaptic activity. However, subsequent work suggested that the generation of action potentials in a cortical neuron does not ensure that ocular dominance modifications will occur after MD. To reconcile these data with the Stent model, Singer (In FO Schmitt and FG Worden (eds) "The Neurosciences Fourth Study Program," Cambridge, MA: MIT Press, pp 1093-1109; 1979) introduced the idea that there is a critical level of postsynaptic activition that must be reached before experience-dependent modifications will occur, and that this threshold is higher than the depolarization required for somatic sodium-spikes.

This hypothesis was recently tested in a study by Reiter and Stryker (Proc. Natl. Acad Sci. 1988). They infused continuously the $GABA_A$ receptor agonist muscimol into striate cortex as kittens were monocularly deprived for 7 days. With the muscimol still present in cortex, they mapped the cortex to determine the extent of activity blockade. They found that all cortical cell responses were eliminated within several millimiters of the infusion cannula, even though LGN fiber activity was readily demonstrated. When the muscimol wore off, they performed an ocular dominance assay in the zone of cortex whose activity had been blocked. They observed an unexpected ocular dominance shift toward the *deprived eye*; that is, most neurons were no longer responsive to stimulation of the retina that had been more active during the period of MD. These data suggest that patterned presynaptic activity can lead to either an increase or a decrease in synaptic strength, depending on whether or not the target neurons are allowed to respond.

The results of this study are inconsistent with the Stent-Singer model because synaptic modifications were observed in the absence of demonstrable postsynaptic activity. However, they are compatible with an alternative theoretical solution to the problem of visual cortical plasticity, developed by Leon Cooper and his associates at Brown University (reviewed by Bear, *et al.*, Science 237: 42; 1987). According to this theory, the efficacy of active synapses increases when the postsynaptic target is concurrently depolarized beyond a "modification threshold", θ. However, when the level of postsynaptic activity falls below θ, then the strength of active synapses decreases. Thus, "effective" synapses are strengthened and "ineffective" synapses are weakened, where synaptic effectiveness is determined by whether or not the presynaptic pattern of activity is accompanied by the simultaneous depolarization of the target dendrite beyond the modification threshold, θ.

An important feature of this model is that the value of the modification threshold is not fixed, but instead varies as a non-linear function of the average output of the postsynaptic neuron [the time over which postsynaptic activity is averaged can be inferred to be in the range of several hours]. This feature provides the stability properties of the model, and is necessary to explain why the low level of postsynaptic activity caused by binocular deprivation does not drive the strengths of all cortical synapses to zero.

Analysis and computer simulation have shown that virtually all of the available data on the experience-dependent modification of striate cortex can be explained by this simple learning algorithm (Bienenstock, *et al.*, J. Neurosci 2: 32; 1982; Clothiaux, Bear and Cooper, unpublished). While this is satisfying in its own right, the critical question remains as to whether this form of synaptic modification has a neurobiological basis. Recall the following three distinctive features of the theory.

(1) The sign of the synaptic modification depends on whether the postsynaptic depolarization is greater or less than θ.

(2) The rate of the synaptic modification depends on the concurrent level of presynaptic input activity.

(3) The value of θ varies with the average activity of the neuron.

This leads to the following 3 questions that we have begun to address experimentally.

(1) When input activity is high, what distinquishes postsynaptic depolarizations greater than θ from those less than θ?

(2) When postsynaptic depolarization is less than θ, what distinquishes high from low input activity?

(3) What is the molecular basis of the sliding modification threshold?

My lecture constitutes a status report on our efforts to answer these questions.

Sweatt, J.D. and Kandel, E.R. MOLECULAR MECHANISMS FOR PRESYNAPTIC FACILITATION AND INHIBITION IN APLYSIA

We have used a simple system to study learning: the defensive withdrawal of gill and siphon in Aplysia. When the animal's siphon is stimulated by a light touch, the gill and siphon contract and withdraw vigorously. The gill- and siphon-withdrawal reflex in Aplysia exhibits both positive and negative modulation. A strong shock to the animal's tail causes facilitation of the gill- and siphon-withdrawal reflex, that is, an increase in the magnitude of the response. A weaker tail shock unmasks an inhibitory component that is present, which is responsible for decreasing gill and siphon withdrawal and which transiently overrides the facilitatory component. The neural circuitry underlying these changes in behavior has been traced, and certain of the neurotransmitters involved have been identified. The gill- and siphon-withdrawal reflex is mediated in part by a monosynaptic reflex arc composed of siphon sensory neurons and gill motor neurons. A significant component of the facilitation of the reflex is due to activation by tail sensory neurons of interneurons that release serotonin presynaptically onto the siphon sensory neuron. The serotonin acts presynaptically to cause increased release of neurotransmitter from the siphon sensory neuron. The inhibitory component is also mediated in part by interneurons, activated by tail sensory neurons, that release the peptide FMRFamide at the sensory neuron-motor neuron synapse. FMRFamide causes inhibition of neurotransmitter release presynaptically.

Based on biophysical and biochemical studies on the siphon sensory neurons, a model for presynaptic facilitation has been outlined. Serotonin, released onto the presynaptic terminal, activates a transmitter-sensitive adenylate cyclase that increases the level of cAMP intracellularly. Cyclic AMP then activates a protein kinase that phosphorylates a number of substrate

proteins, including a K^+ channel protein (or a protein associated with it). The phosphorylation causes closure of the K^+ channels (called S channels), reducing one of the K^+ conductances that normally repolarize the action potential. This has three consequences: it depolarizes the cell, it increases the excitability of the neuron and prolongs the action potentials so as to allow more Ca^{++} to flow into the terminals, and, finally, it contributes to increased release of transmitter (see Kandel and Schwartz, Science 218:433-443, 1982, for a review). Independent of "S" K^+-channel closure, serotonin also alters the handling of Ca^{++} within the sensory neurons, which leads to a rise in intracellular free Ca^{++} (Boyle et al., Proc. Natl. Acad. Sci. USA 81:7642-7646, 1984). This rise in free Ca^{++} may be important for the mobilization of transmitter in the face of continued transmitter release.

Recent evidence has allowed the formulation of a model for the molecular basis of presynaptic inhibition. FMRFamide acts presynaptically to cause inhibition of neurotransmitter release, the mirror image of presynaptic facilitation. When applied to a sensory neuron, FMRFamide produces a transient hyperpolarization, an increase in membrane conductance, and a narrowing of the action potential. To produce this effect, FMRFamide alters several macroscopic currents, including that of the "S" K^+ channel. In contrast to the effect of serotonin, FMRFamide causes opening of the "S" K^+ channel. Recent work by Piomelli et al. (Nature 328:38-43, 1987) has suggested a molecular mechanism for this effect, that is, that FMRFamide causes production in the sensory neurons of lipoxygenase metabolites of arachidonic acid, which act as second messengers to mediate presynaptic inhibition and opening of the "S" K^+ channel.

One interesting attribute of the neural circuit described above is that inhibition overrides facilitation. This effect is observed at the

behavioral, cellular, and biophysical level. For example, the simultaneous application of FMRFamide and serotonin to sensory neurons yields a net preesynaptic inhibition. This observation prompted us to ask how this override was manifest at the molecular level.

How does FMRFamide bring about an override of the serotonin-stimulated cyclic AMP cascade? One possibility is that FMRFamide, acting through an inhibitory G protein, could cause inhibition of adenylate cyclase and thus reversal of the effect of serotonin. However, Ocorr and Byrne (Neurosci. Letts. 55:113-118, 1985) had previously found that FMRFamide could not cause a decrease in cAMP levels in sensory neurons, suggesting that FMRFamide is unable to cause inhibition of adenylate cyclase. We confirmed their results and also found that FMRFamide was unable to diminish serotonin-stimulated cAMP production in sensory neurons. These findings suggest that FMRFamide is unable to cause inhibition of adenylate cyclase in sensory neurons.

A second possibility is that FMRFamide-induced inhibition of the serotonin/cyclic AMP response could be at the level of regulation of protein phosphorylation. For example, FMRFamide might elicit phosphatase activation or inhibition of the cyclic AMP-dependent protein kinase in order to block the effects of serotonin. To address this possibility, we developed an intact sensory neuron protein kinase assay, based on ^{32}P-phosphate labeling and quantitative two-dimensional gel analysis of ^{32}P-labeled proteins. We found in initial studies that a single pulse of serotonin or of cell-permeable cyclic AMP analog to intact sensory neurons causes an increase in phosphorylation of a specific set of proteins. Serotonin or cyclic AMP increase the level of phosphorylation of 17 proteins, ranging in molecular weight from 16,000 to 60,000 and in pI from 4.5 to 7.5. No proteins have their phosphorylation decreased in response to serotonin or cyclic AMP. These data

suggest that serotonin is capable of causing activation of the cAMP-dependent protein kinase in sensory neurons. The alteration of a relatively large number of proteins also suggests that the action of serotonin is not limited to the "S" K^+ channel but extends to a wide variety of biochemical processes in sensory neurons.

How does FMRFamide act on this set of phosphorylation events? When applied by itself, FMRFamide decreases the level of phosphorylation of 9 of the 17 proteins whose phosphorylation increases upon serotonin or cAMP stimulation. When presented together with serotonin or cyclic AMP analog, FMRFamide completely overrides the actions of serotonin or cyclic AMP. Thus, these data are consistent with the hypothesis that FMRFamide leads to kinase inhibition or phosphatase activation in sensory neurons. We are presently undertaking in vitro biochemical studies to try and distinguish between these possibilities. Finally, these findings are interesting in that FMRFamide shows, at the level of protein phosphorylation, the inhibitory override observed at both the behavioral and cellular level.

NEURAL MECHANISMS OF EXPERIENCE-DEPENDENT SELF-ORGANIZATION OF CEREBRAL CORTEX

W. Singer

Remodelling of already established neuronal connections is a common process during the development of the central nervous system. Once neuronal connections are established they remain malleable for a critical period, during which some of them become stabilized and others removed. From a certain developmental stage onwards this pruning of connectivity is controlled by the activity patterns conveyed in the malleable pathways. In the visual cortex such activity-dependent selection processes extend into postnatal life. Consequently, sensory experience influences the development of cortical circuitry. Monocular deprivation is the most thoroughly studied paradigm for the investigation of vision-dependent long-term modifications of synaptic connectivity. It leads to a rapid inactivation of the deprived pathways. Variations of this paradigm have suggested that connections stabilize if pre- and postsynaptic elements are frequently active in temporal contiguity while they destabilize when pre -and postsynaptic activity patterns are poorly correlated. Experiments with rapidly alternating monocular occlusion suggest that this matching interval has a duration of approximately 300-400 ms. (Altmann et al., 1987). Chronic recordings from individual neurones show that changes of excitatory transmission can be induced with brief exposure (<2 h) but manifest themselves only after several hours, suggesting the existence of a consolidation period. Lesion experiments indicate further that change induction requires co-operativity between retinal activity and additional internally generated signals (for review see Singer, 1987). This co-operativity seems to be required to reach the activation threshold of postsynaptic Ca2+ channels, Ca2+ entry into postsynaptic profiles being most likely one of the change-inducing signals (Geiger and Singer, 1986). Activation of the NMDA receptor whose voltage sensitive ionophore is capable of "evaluating" contiguity of pre- and postsynaptic activity is crucial for the mediation of use-dependent modifications. Its blockade with APV abolishes all experiential effects on circuitry (Kleinschmidt et al., 1987). Furthermore, the noradrenergic and the

cholinergic projections to striate cortex have been shown to have permissive gating functions in use-dependent plasticity (Bear and Singer, 1986). Correspondingly, long-lasting modifications of synaptic transmission can be induced rapidly if the neurotransmitters norepinephrine, acetylcholine and NMDA are applied directly to cortical neurones while the corresponding retinal afferents are activated with light stimuli. Complementary studies in cortical slices support the notion that the activation of the NMDA mechanism is necessary for the induction of long-term modifications of excitatory transmission (Artola and Singer, 1987). The hypothesis that Ca2+ channels play a role in developmental plasticity is supported by the good correlation between the time course of cortical malleability and the gradual redistribution of Ca2+ channels during development (Bode-Greuel and Singer, 1988). In these respects use-dependent plasticity during development has numerous properties in common with activity-dependent malleability of mature neuronal circuits. Examples are long-term modifications of synaptic efficacy in the hippocampus, the cerebellum and the cerebral neocortex. This suggests that some of the mechanisms underlying developmental plasticity are preserved in the adult nervous system and may subserve adaptive functions associated with learning.

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JEUDI 7 JUILLET 1988

9 h - 12 h 30

DEVELOPPEMENT DU SYSTEME NERVEUX CENTRAL

DEVELOPMENT OF CENTRAL NERVOUS SYSTEM

NEURAL CREST

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The neural crest is a structure, phylogenetically restricted to vertebrate embryos, that forms at the lateral margins of the neural plate and materialises at around the time when closure of the neural tube occurs. As the source of the entire peripheral nervous system (with the exception of certain cranial ganglion cells), the neural crest constitutes a relatively simple model system that can be used with profit to study the initial stages of neural development. It is also fascinating to embryologists who are not specifically interested in the nervous system, but are seeking replies to fundamental questions concerning potentiality, determination, cell lineages, plasticity and the mechanisms underlying differentiation processes. Although it has already been subjected to interactions resulting in a restriction of its developmental capacity (it is formed after gastrulation has organised the three primary germ layers), the neural crest retains a striking pluripotentiality, giving rise not only to the diverse categories of neurons that are found in the sensory, autonomic and enteric nervous systems and to Schwann and satellite cells, but also to a variety of other derivatives that include melanocytes, certain endocrine and paraendocrine cells and connective, skeletal and muscular tissue in the head. The question naturally arises as to how such a multiplicity of cell types arises from a structure that is apparently homogeneous.

An in vitro approach, involving the construction of chimaeric embryos in which neural crest cells and their derivatives are indelibly labelled, has revealed that the differentiation of neural crest-derived cells is largely conditioned by the embryonic microenvironment they encounter during and/or at the end of their migration from the neural axis. The neural crest is, in fact, essentially a homogeneously pluripotential population and, although all its

developmental possibilities are not normally implemented at each level of the neural axis, they can be realised experimentally by exposing the cells to appropriate environmental influences. One therefore wishes to know whether this global pluripotentiality reflects an analagous situation at the level of each individual cell or whether the neural crest is, in reality, a mosaic structure, composed of different categories of cells, each of which is committed to following a particular pathway of differentiation. Do environmental factors play an instructive role , imposing a particular developmental outcome on cells that have multiple options open to them, or do they act rather by selectively promoting the survival and multiplication of specific sub-sets of committed cells whose subsequent differentiation would follow an intrinsically determined programme? Although it seems clear that by no means all developmental decisions are predetermined when neural crest cells begin to leave the neural tube, recent work suggests that the migrating neural crest is composed of a population of cells that are heterogeneous in their developmental potentialities. This has been demonstrated directly by growing neural crest cells individually in vitro under permissive conditions. Numerous clones were obtained that differed greatly in size and in the range of phenotypes that they expressed. The majority contained several different cell types (in a variety of combinations), indicating that they had arisen from pluripotent (but different) precursors; however, some were composed of only one kind of derivative (e.g. neurons or Schwann cells), suggesting that the crest cell from which they derived was unipotential. The data are consistent with a model in which differentiation potentials become progressively (but not necessarily simultaneously) restricted as neural crest progeny migrate and accumulate at the sites of peripheral ganglion formation. Since there is no experimental justification for the supposition that differently committed cells are channelled along different migration pathways, the implication is that the cells which ultimately accumulate to form the different kinds of peripheral ganglia will include several types of precursor only some of which are destined to differentiate in any given site.

An essential role of the environment would thus be to select, from a population of variously committed progenitors, only

those that are appropriate for that particular location. An illustration is provided by the formation of sensory dorsal root ganglia, which develop from a mixed population including precursors of sensory and of autonomic type. Precursors committed to the sensory lineage specifically require, for their survival, one or more factors found in an extract of neural tube. The precursors with autonomic potentialities that settle in the sites where sensory ganglia form survive there, without differentiating, throughout and beyond embryonic life, as a discrete population of non-neuronal cells. Their development into neuron-like cells expressing an autonomic (catecholaminergic) phenotype can be triggered in vitro by exposure to insulin or insulin-like polypeptides. It is suggested that site-specific phenotypic expression during ontogeny of neural crest may in part reflect local differences in distribution of growth factors and/or their receptors.

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THE HOMEOBOX AND NEUROGENESIS IN *DROSOPHILA*

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The **body plan** of *Drosophila* is specified basically by three classes of control genes; maternally expressed genes, segmentation genes and homeotic genes. These three classes of genes specify the body plan in three consecutive conceptual steps (Gehring, 1987). First, **maternally expressed control genes** determine the antero-posterior and the dorso-ventral polarity of the egg, i.e. the spatial coordinates of the future embryo. Second, the **segmentation genes** generate a repetitive pattern of body segments by determining the number and polarity of the body segments and third, the homeotic genes generate a sequential pattern of body segments by specifying the identity of the individual segments and their sequence. The **homeobox** (McGinnis et al., 1984a and b; Scott and Weiner, 1984), a small DNA segment of 180 bp, has been found not only in most homeotic genes, but also in several segmentation genes and at least in two maternally expressed control genes. The homeobox has allowed the cloning of over twenty such control genes and to analyze their mechanism of action at the molecular level. It encodes a protein domain designated as the homeodomain that constitutes only part of the much larger homeo proteins. Following its discovery in *Drosophila*, the homeobox was not only found in arthropods and their ancestors but also in vertebrates including man. The first indication of the possible function of the homeodomain came from comparative protein sequence analysis, which revealed a small but significant degree of homology of 20-25% to the yeast mating-type proteins MATa1 and MATα2 (Shepherd et al., 1984). The MAT genes of yeast are known to encode gene regulatory proteins. These proteins bind to specific DNA sequences in the upstream regulatory regions of those target genes which they regulate. These homologies suggest a similar function for the homeodomain. In order to test this hypothesis we have expressed the homeodomain as a peptide of 68 amino acids in an expression vector in *E.coli* (Müller and Gehring, unpubl.). *In vitro* DNA binding studies indicate that the purified homeodomain binds selectively to specific DNA sequences (Affolter, Müller & Gehring, unpubl.). The *in vitro* binding sites correspond to those found for the entire homeo proteins.

The RNA and protein products of the homeobox-containing genes have been localized by *in situ* hybridization and immunolocalization respectively. All homeoproteins analyzed so far are localized in the nucleus which is compatible with their proposed function in gene regulation. The localization of the RNA transcripts and the proteins clearly reflect the three conceptual steps involved in the establishment of the body plan. Shortly after fertilization, during the early nuclear divisions, the maternally expressed gene products of *caudal* and *bicoid* form a concentration gradient of RNA and protein along the antero-posterior axis. Segmentation genes like *fushi tarazu (ftz)* form a repetitive pattern of stripes perpendicular to the antero-posterior axis. Finally, the homeotic genes form a single stripe comprising those segments which they specify. For example, the *Antennapedia* gene *(Antp)* is expressed primarily in the second thoracic segment (T2) and parts

of T1 and T3. The hypothesis that *Antp* specifies T2 is supported by the finding that mutations that lead to a loss of function of the *Antp* gene lead to a loss of T2 and its replacement by T1, whereas dominant gain of function mutants which affect the spatial and temporal pattern of *Antp* expression lead to the formation of additional T2 structures in the head region, i.e. the transformation of antennae into middle legs. To test the hypothesis that *Antp* indeed specifies T2 structures, we have fused a cDNA encoding the *Antp* protein with a heat inducible promoter and reintroduced this construct into the germ-line of normal flies (Schneuwly et al., 1987). By a brief heat shock at 37°C the *Antp* protein can be induced in all cells of the transgenic flies. If the *Antp* protein is induced at a specific time (during the early third larval stage) the antennae on the head of the fly, indeed become transformed into middle legs. This is the first successful redesigning of the body plan of the fruit fly.

At later developmental stages, most of the homeobox-containing genes become expressed in specific areas of the **central nervous system**. This observation applies not only to *Drosophila* but also to vertebrates. The insect nervous system arises from an undifferentiated sheet of the ventral ectoderm (see Doe et al., 1985). Each segment contains about 500 neurons many of which can be identified according to their morphology and synaptic connections. The generation of neuronal specificity occurs in two steps. First, in each segment, the morphologically uniform ectoderm differentiates into a defined array of neuronal precursor cells, namely, neuroblasts, midline precursors and non-neuronal cells. Second, each neuroblast within this array divides asymmetrically to generate a characteristic chain of progeny, called ganglion mother cells, each of which divides once more symmetrically to produce pairs of sibling neurons. In contrast, the midline precursors divide only once to generate two neuronal progeny. Laser ablation experiments show that any cell in the neuroectoderm can become a neuroblast, but once a cell enlarges to become a neuroblast, it inhibits its neighbours to do so and causes them to differentiate into non-neuronal cells. Each neuroblast appears to be assigned its individual identity according to the position at which it enlarges within the neuroectodermal sheet. Determined by its position, each neuroblast goes on to generate its characteristic family of ganglion mother cells by an invariant cell lineage. Thus, the fate of each individual neuron is determined both by its lineage and interactions with other cells.

Since the segmentation gene *ftz*, which also contains a homeobox, is transiently expressed in a specific subset of neuronal precursors, neurons and glial cells, we analyzed its functional role in the developing central nervous system. Previous gene transfer experiments had indicated separate control regions in the 5´ flanking sequences of the *ftz* gene responsible for the early expression in the stripes, reflecting the body plan, and the subsequent expression in the nervous system (Hiromi et al., 1985; Hiromi & Gehring, 1987). In particular, a "neurogenic element" was identified which is indispensable for the expression in the central nervous system. On the basis of this information, an artificial mutant was constructed by gene transfer which lacks only the neurogenic element. Such

mutant embryos express *ftz* normally in the seven stripes but not in the central nervous system (Doe et al., 1988) and die before hatching from the egg shell. Injection of the fluorscent dye lucifer yellow into individual neurons indicates that in the absence of *ftz* expression some neurons appear normal, whereas the RP2 neuron extends its growth cone along an abnormal pathway, following its sibling RP1 indicating a transformation in neuronal identity. These experiments strongly suggest that homeobox-containing genes also control cell fate during neurogenesis.

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TRANSPLANTATION D'OLIGODENDROCYTES DANS LE SNC DES MAMMIFERES

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INSERM U 134 - HOPITAL DE LA SALPETRIERE - 75651 PARIS CEDEX 13

Si la technique de la transplantation intracérébrale de cellules neurales a été largement utilisée au cours de ces 15 dernières années, peu d'études concernent le devenir des oligodendrocytes transplantés. En effet, il fallait pour cela être en mesure de distinguer les oligodendrocytes implantés de ceux de l'hôte ou la myéline formée par les cellules implantées de la myéline de l'hôte. Dans cette perspective, nous avons mis au point plusieurs modèles permettant de distinguer soit les cellules, soit leurs produits de synthèse.

J'analyserai ici le problème de la remyélinisation d'une lésion de démyélinisation dans la moelle épinière de la souris adulte par transplantation d'oligodendrocytes immatures. Dans cette série expérimentale nous avons utilisé le modèle shiverer.

Le modèle shiverer :

Shiverer est une mutation autosomique récessive de la souris. Cette mutation située sur le chromosome 18 (18) est une délétion de 5 des 7 exons. L'animal homozygote shiverer (shi/shi) présente de ce fait une absence totale de PBE l'une des protéines essentielles de la myéline centrale. Corrélativement à ce défaut biochimique la myéline centrale observée au microscope électronique (9, 17) est complètement dépourvue de ligne dense majeure (LDM).

Grâce à ces défauts, si l'on greffe dans le cerveau d'un animal shiverer des oligodendrocytes immatures issus d'une espèce normale, la myéline formée par les oligodendrocytes implantés pourra être distinguée de la myéline de l'hôte par immunohistochimie en utilisant un anticorps anti-PBE ou à l'observation au microscope électronique.

Matériel et méthodes

Des animaux shiverer adultes (1 à 2 mois) reçoivent dans la moelle épinière une injection de 2 µl de lysolecithine à 1 % en solution dans du liquide physiologique. Le point d'injection est marqué au charbon. Par ce moyen, on obtient une démyélinisation locale extrêmement rapide (14, 11) au niveau de l'un des cordons postérieurs. En même temps on transplante à une distance de 1 à 3 espaces intervertébraux en direction caudale ou rostrale (3 à 8 mm) un fragment de CNS de souris normale nouveau-né contenant donc des oligodendrocytes immatures. Selon une technique déjà décrite (10,12,13,16) . Les animaux témoins shiverer de même âge reçoivent soit l'injection de lysolecthine seule, soit une injection du même volume de liquide physiologique. Par ailleurs des souris témoins normales (C3H/CWV) de même âge reçoivent une injection démyélinisante de lysolpicithine. Les animaux sont sacrifiés entre 2 et 94 jours après l'opération. Ils sont fixés par perfusion intracardiaque de 150 ml d'un fixateur contenant 4 % de paraformaldehyde et 1 % de glutanaldehyde dans le Sörensen 0,1M (pH 7,2). La moelle est ensuite disséquée puis fixée pendant 6 h par immersion dans le même fixateur, lavée très longuement dans le tampon, puis fixée à nouveau par immersion pendant 30 minutes dans l'acide osmique. Les échantillons sont ensuite déshydratés et inclus dans l'epon. Après examen des coupes semi-fines, les coupes ultrafines (600 Å) sont contrastées par l'acétate d'uranyl et le citrate de plomb et exminées au microscope électronique.

Résultats .

Les témoins : La moelle des souris shiverer injectées avec la lysolecithine le processus de remyélinisation est tout à fait comparable à la remyélinisation chez la souris normale après la même injection. Après 2 mois, la lésion est pratiquement complétement réparée par des cellules de l'hôte. La myéline de réparation est donc normale (avec LDM) chez la souris normale, de type shiverer chez la souris shiverer.

Après injection de liquide physiologique, une lésion discrète peut être observée qui n'est pas spécifiquement une lésion de démyélinisation.

Les résultats : obtenus après démyélinisation chez la souris shiverer adulte et transplantation à distance d'un greffon contenant des oligodendrocytes normaux immatures sont regroupés dans le tableau I.

La remyélinisation spontanée par les oligodendrocytes de l'hôte est visible dès le 9e jour après l'intervention. En revanche, une remyélinisation par les oligodendrocytes du greffon (LDM+) est observée pour la première fois 38 jours après la greffe. A partir du 67e jour, dans tous les cas, la remyélinisation de la lésion est mixte. shiverer et normale. Des oligodendrocytes immatures contenus dans le greffon ont donc franchi la distance entre le greffon et la lésion (3 à 8 mm) pour participer à la réparation de la lésion (11).

Conclusion et discussion.

Un travail récent de Blackemore (7) indique que des oligodendrocytes transplantés peuvent remyéliniser une lésion de démyélinisation. Cependant, l'auteur transplante les cellules dans la lésion après une irradiation qui supprime la remyélinisatin spontanée.

Dans le présent travail, nous montrons que des oligodendrocytes immatures ou des cellules précurseurs sont capables de migrer à travers un tissu cérébral adulte en direction d'une lésion de démyélinisation. Small et al (18) ont prouvé la migration de cellules précurseurs des oligodendrocytes
qui colonisent progressivement le nerf optique de rat du cerveau vers la rétine. D'autre part, des expériences de notre groupe montrent que des oligodendrocytes embryonnaires ou nouveau-nés, donc probablement à l'état de précurseurs, transplantés dans le cerveau de la souris shiverer nouveau-né (1,10,13,16) migrent sur de longues distances et participent au processus de myélinisation de l'hôte en compétition avec ses propres oligodendrocytes. Dans le cas de lésion de démyélinisation chez l'animal adulte, une remyélinisation spontanée intervient. Elle est le fait d'oligodendrocytes ou de cellules de Schwann (2,15) . Les transplantations de cellules de Schwann dans différentes conditions ont prouvé que ces cellules peuvent participer à la remyélinisation de lésions de démyélinisation quand elles sont transplantées dans la lésion (2,3,4,5,6,8). Elles migrent cependant peu dans le cerveau de l'hôte adulte.

Le présent travail montre que des cellules contenues dans le greffon issu d'un animal nouveau-né (qui sont donc probablement en majorité des cellules précurseurs) peuvent se mouvoir dans le parenchyme cérébral adulte et que ce mouvement se fait en direction de la lésion.

Deux questions se posent : 1- Quels sont les mécanismes qui font que les cellules du greffon se déplacent en direction de la lésion ?
2- Lors de la remyélinisation spontanée, quelles sont les cellules qui se déplacent vers la lésion et sont responsables de la remyélinisation ?
Ces deux problèmes sont actuellement à l'étude.

Acknowledgements

Ce travail a été réalisé grâce au CNRS à l'INSERM, à l'ARSEP, à la Multiple Sclerosis Society et à la Fondation pour la Recherche médicale.

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Tableau I : Résultats observés chez 16 souris Shiverer démyélinisées et greffées
+/- : Remyélinisation spontanée en cours

N°	Nombre de jours entre l'opération et la perfusion	Distance entre la lésion de démyélinisation et la greffe nombre d'espaces intervertébraux	Remyélinisation	
			par des cellules de l'hôte (LDM -)	par les cellules greffées (LMD+)
296	9	1	+/-	-
299	14	1	+/-	-
226	16	1	+/-	-
235	24	1	+/-	-
306	26	1	+/-	-
288	33	1	+	-
291	36	1	+	-
312	38	1	+	+
295	39	1	+	-
298	44	1	+	-
326	67	2	+	+
327	67	2	+	+
253	76	1	+	+
265	94	2	+	+
264	94	3	+	+
266	94	2	+	+

JEUDI 7 JUILLET 1988

14 h 30 - 18 h

SIDA : FAITS ET THEORIES

AIDS : FACTS AND THEORIES

Theory of an Immune System Retrovirus

Leon N Cooper

The retrovirus human immunodeficienty virus (HIV; formerly known as human T-cell lymphotropic virus type III/lumphadenopathy-associated virus, HTLV-III/LAV) has been implicated as the agent responsible for the acquired immune deficiency syndrome. This virus infects T4-positive (helper) T cells of the immune system, and other cell types as well, and has shown certain peculiarities in its life cycle. In particular, the simultaneous infection of HIV and other viruses--or could be the result of a more general interaction between HIV and any other agent that stimulates the immune system.

In what follows, using simple models for virus growth and lymphocyte expansion, I contrast the growth of a "normal" virus with what I call an immune system retrovirus (ISRV): a retrovirus that infects T4-positive (helper) T cells of the immune system. I show that remarkable interactions with other infections as well as strong virus concentration dependence are general properties of ISRV. The equations for virus growth and lymphocyte expansion are treated here approximately. A more detailed analysis will be presented elsewhere.

To what extent this account of ISRV provides an accurate description of the life cycle of HIV is, of course, an experimental question.

The argument is based on the following assumptions: an immune system retrovirus (ISRV) is a retrovirus that infects T_4 (helper) cells of the immune system. The retrovirus can invade the cell upon proper receptor contact but is integrated into cell DNA only in the mitotic phase. Since the mitotic phase is induced by a binary recognition event, integration and (possibly) virus reproduction requires that the invaded target T cell interact with the appropriate B-cell-processed antigen complex.

In contrast with a normal virus, for ISRV there is a complicated pattern of growth regions depending critically on the concentration of ISRV, B and T cells reactive to ISRV and on activity of the immune system due to other infections or natural turnover.

For a first-time low concentration infection with low $B_y(o)$ and $T_y(o)$ populations (with little or no other stimulation of the immune system) ISRV is produced very slowly since most of the T_4 (helper) cells infected will not be stimulated to reproduce. Thus one would expect some antibody

response but low virus growth. Since the level of ISRV antibody was well as the number B_y and T_y cells determine the rapidity of immune system response immunization to the virus for this situation seems possible. This is consistent with puzzling presence of ISRV Ig in so many individuals who, in spite of the extraordinarily rapid production rate of HTL VIII, show no symptoms.

A further infection by ISRV (as long as Y_0 ia not too large and possibly also as long as B_y and T_y are not too large - since ISRV growth rates depend on these quantities) if not accompanied by a rapidly growing 'normal' infection could then be controlled by the immune system so that the individual would appear to be immunized to ISRV.

If ISRV comes in several genetic variations (each of which contain a site reactive to the same Ig) further infection by a new variety of ISRV after an initial controlled infection (if not accompanied by a rapidly growing normal infection) could be controlled by the immune system so that it would not appear as virus in the individual. Therefore an individual already showing virus (likely in an ISRV chronic state) will in effect be immunized to other strains of the virus so that one would not be likely to find more than one very distinct genetic variation in a single individual.

However, for an ISRV infection simultaneous with another rapidly growing virus or other infection, [The second infection could be a chronic illness such as malaria; it could occur at the same time as the ISRV infection or at some later time when the individual still has ISRV invaded T cells.] there is rapid growth of ISRV resulting almost certainly in destruction of immune system response.

Thus, in striking contrast to a normal virus, no level of initial B and T memory cells can give complete protection since simultaneous 'normal' and ISRV infection (if one is not already immunized to the normal infection) leads to catastrophically rapid ISRV growth rates, destruction of T_4 cells and no possible immune system control. Because of this each repeated infection of ISRV exposes the individual to this risk in spite of his level of 'immunization'.
Once the concentration of infected T_4 cells grows large enough compared to the concentration of healthy T_4 cells, since the growth rate of ISRV depends on concentrations of B_y cells, it appears that, again in contrast with a normal virus, for ISRV <u>increasing the level of B_y becomes counter-productive.</u>

It is possible that for an initial controlled infection the virus can lurk unintegrated or possibly integrated and non-producing (if reproduction, as has been suggested, requires some other signal) in those T_4 helper cells not stimulated to reproduce. Thus even with an effective immune system response resulting in appropriate B and T cells and Ig, virus can continue to exist in infected T_4 cells. If these cells are stimulated by some other infection (or even a subsequent IRSV infection) virus can again be produced, again entering the blood so that the infection appears once more. In the absence of further infection the virus will continue to exist in infected T_4 cells until these cells die naturally. Thus one might speculate that an otherwise healthy individual exposed to ISRV could show an immune system response (ISRV Ig) no easily visible symptoms, and with no further infection, might rid himself of the virus in the time due to the natural turnover of T cells.

When a sufficient number of T_4 cells are infected by ISRV an intermediate siutation could result in a chronic infection: continual low production of ISRV and reinfection of T_4 cells due to slow stimulation of the immune system by ISRV itself but no catastrophic growth of ISRV.

One of the consequences of these arguments is that a high concentration of Ig reactive to ISRV can serve to control the ISRV infection while a high concentration of T_y and B_y (the natural source of ISRV reactive Ig) works in the opposite direction since it increases the ISRV growth rate. This suggests that increasing the level of Ig reactive to ISRV while at the same time dampening immune system activity would aid in controlling the growth of ISRV. [One would, of course, have to deal with the growth of other infections.]

In the above analysis no assumptions have been made concerning biological co-factors and/or latency or incubation periods. If these exist they would modify but not negate our discussion. However, a consequence of our arguments is that there can be delays between infection and syndrome even though there is no intrinsic prolonged latency period; any immune system stimulant acts as a co-factor.

As one application of these ideas consider Kasposi's sarcoma. Among the earliest observations in AIDS patients was the increased virulence of this sarcoma. It has further been observed that this increase in virulence is correlated to the presence of the HTL VIII antibody.

It is believed that Kaposi's sarcoma is caused by a virus (a virus independent of HTLV III) and it seems likely that the spread of this sarcoma is mediated by the virus as much as by migration of malignant cells.

Following the above arguments, this can be analyzed as follows. In a patient, not infected with ISRV the spread of Kaposi's sarcoma is inhibited by normal immune system function -- the immune system preventing the spread of the virus. In the patient infected with HTL VIII, the virus that produces the sarcoma acts as a 'normal' - virus described above. The stimulation this virus provides for the immune system increases the growth of the ISRV. This destroys the capacity of the immune system to respond thus allowing the spread of the normal virus resulting in the increased virulence of Kaposi's sarcoma. [This, of course, would also be true for other infections spread by 'normal' viruses.]

In this case, we may be seeing the interaction of the immune system retrovirus with a normal virus that would under ordinary circumstances be controlled by the immune system. However, the interaction of these two viruses results in destruction of the immune system and the increased virulence of a normally controllable dissease.

VENDREDI 8 JUILLET 1988

9 h - 12 h 30

SIDA : FAITS ET THEORIES

AIDS : FACTS AND THEORIES

Autoimmunity and AIDS

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The importance of the immunologic system in protection against infectious organisms, inflammation, autoimmunity, tumor immunosurveillance and the pathogenesis of many diseases has become more and more recognized during the past decades. The basic principle of the immune system is to defend the organism against foreign pathogens while not responding adversely to self-components. It must distinguish "self" from "non-self". For this purpose specific recognition systems allow cooperation between T-lymphocytes and B-lymphocytes and monocytes. In turn, cytokines play an important role in regulation of immune cascades. Interleukins control activation and proliferation of lymphocytes whereas the main activity of gamma interferon, a product of activated T-lymphocytes, is to prime monocytes for antimicrobial and tumoricidal activity (1).
Interaction of genetic and environmental factors may, under certain circumstances, bring about that the discrimination of self from non-self can fail and the T-cell and/or the B-cell mechanisms become autoreactive.

Neopterin

D-erythro-neopterin is biosynthesized from guanosinetriphosphate and is a product from human macrophages on stimulation with gamma interferon (2). The concentration of neopterin is easily measured in urine, serum, synovial or cerebrospinal fluid and reflects sensitively the preactivation status of the cell mediated immunity. Increased neopterin levels indicate rejection episodes in allograft recipients or viral infections early, and they parallel the course of autoimmune disorders. T-cells release gamma interferon whenever they

recognize altered-self or non-self major histocompatibility complex determinants in cooperation with antigen presenting cells (APC), e.g. monocytes. This event is not necessarily accompanied by proliferation and cytotoxicity of cells. Thus, neopterin levels are representative only for the afferent site of cellular immune response.

Autoimmune Diseases

In general, autoimmune phenomena are closely linked to high neopterin levels indicating increased production of gamma-interferon (2). Although the etiology of autoimmune disorders still remains unknown neopterin data fit into the scheme that loss of immune tolerance leads to self-destruction which is accompanied by release of lymphokines (Figure 1). A crucial role of gamma-interferon in triggering autoimmune phenomena is suspected.
In rheumatoid arthritis as a paradigm for autoimmune disorders neopterin was shown to be more sensitive than other routine diagnostics as quantitation of e.g. rheumatoid factors and erythrocyte sedimentation rate. Neopterin levels reflect the course of disease most accurately (3). Higher neopterin concentrations in synovial fluid than in serum of patients indicate local production at the site of the active immunological process (4). In addition, neopterin levels are powerful in differentiating rheumatoid arthritis from non-autoimmune degenerative disorders with similar clinical symptoms, e.g. osteoarthrosis.

Infections with Viruses, Parasites and Intracellular Bacteria

Life cycles of viruses, parasites and certain bacteria (e.g. mycobacterium tuberculosis, mycobacterium leprae) include at least transient integration of the pathogen into the host cell. These pathogens replicate within the host cell and specific proteins of these pathogens are in parallel expressed on the host cell surface. In addition, pathogens may penetrate the cell surface back to the blood stream. In both situations foreign protein structures are recognized by immune cells, T-lymphocytes and monocytes. In presence

of self surface structures a cell mediated immune response is initiated (Figure 2). Immune cascades involve interplay between cells and release of lymphokines, gamma interferon being one of the first mediators. Therefore, increase of neopterin levels is a first sign in virus infections which starts days before antibodies as a result of B-lymphocyte activity become measurable in the circulation (2). During defense operations of cell mediated immunity infected host cells are destroyed. Successful immune response clears the organism only from cells in which the pathogen is reproduced but not from cells where the pathogen is latently present without reproduction. These latent infections cause acute disease any time when immunosurveillance is inproper.

Infection with Human Immunodeficiency Virus (HIV)

HIV uses the CD4-molecule as receptor for its entry and therefore infects CD4-positive cells. T4 lymphocytes are successively eliminated during the course of HIV infection. HIV infects other CD4 positive cells also, e.g., monocytes, and these may serve as important viral reservoirs in the host but T4 lymphocytes are the key population for generating and regulating the immune response. The selective destroyment of these cells has fatal consequences so that protection against invading pathogens is no longer established. Stimulation of target cells increases their susceptibility to be infected with HIV, and immunologic activation of infected cells triggers replication of HIV and cell death. These observations may account for succesive steps in the pathogenesis of AIDS: Latently infected T-cells will be induced to HIV replication via immunologic activation which often occurs in AIDS risk groups linked to behaviour but also banal secondary infections may serve as cofactors (5). Replicating cells express HIV-proteins, e.g., gp 120, on their cell surfaces and become immunogenic. A cellular immune response against these cells (Figure 3) is induced similar to common viral infections and destroys T4 cells. In addition, those immune cells processing HIV-infected cells are highly susceptible to be themselves infected by HIV because they are activated. Moreover, the release of lymphokines within this procedure may induce HIV

replication in other latently infected cells and support dissemination of HIV within the host's organism. In addition, cells bind viral proteins instead of whole virus at their surface, e.g., gp 120 at the CD4 molecule, and will become immunogenic.
The crucial importance of cellular immune activation in regulation of HIV infection can be demonstrated by neopterin levels. Neopterin concentrations not only parallel the course of HIV infection, they are inversely correlated to numbers of CD4+ T-cells and ratios of T-cell subsets, T4/T8. Moreover, higher neopterin levels are significantly predictive for disease progression (2). Thus, the presence of gamma interferon is linked to HIV reproduction in patients. The failure of T-cells to produce gamma interferon in vitro is, therefore, due to continuous exposure of cells to this lymphokine in vivo.

Autoimmunity and Infection with HIV

Cellular immune defense mechanisms depend on the recognition of cells with non-self structures in the presence of self-antigens. As shown (Figure 2 and 3), successful immune reaction comprises destruction of modified host cells also in case of infections with common viruses and also with HIV. Therefore, immunopathology of viral infections and also of HIV infection is somewhat similar to autoimmune diseases. In addition, the cell types involved as well as the mediators produced and the clinical and immunological findings are almost the same in the diseases discussed. Differences in the kinetics are apparent. Once infected with HIV the organism cannot get rid of the immunogen as it is similar with most autoimmune disorders, and more quiescent disease states are followed by disease progression. Persistence of T-cell activation and large amounts of gamma interferon may even trigger autoreactivity which basically means that discrimination of self from non-self fails and self-components are attacked by T-cells, and or B-cells, and/or macrophages in HIV infection.

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Legends to Figures

Figure 1: Simplified Model of Cellular Immune Response in Rheumatoid Arthritis: Cytotoxic T-cells (T_C) erroneously recognize antigens of cartilage cells as "non-self" in presence of major histocompatibility complex (HC) determinants. Immune cascades via interaction of antigen presenting cells (APC), e.g. monocytes, with T_4-helper/ inducer cells (T_H) are induced. Release of cytokines including interferon gamma (IFN-γ) and destruction of cartilage cells by T_C is initiated. Neopterin levels are increased in patients.

Figure 2: Simplified Model of Cellular Immune Response in Viral Infection: Cytotoxic T-cells (T_C) recognize e.g. Epstein-Barr-Virus-(EBV)-antigens on infected B-cells as "non-self" in presence of major histocompatibility complex (HC) determinants . Immune cascades via interaction of antigen presenting cells (APC), e.g. monocytes, with T_4-helper/inducer cells (T_H) are induced. Release of cytokines including interferon gamma (IFN-γ) and destruction of infected B-cells by T_C is initiated. Neopterin levels are increased in patients.

Figure 3: Simplified Model of Cellular Immune Response in HIV Infection: Cytotoxic T-cells (T_C) recognize HIV-antigens on HIV-infected T_4-cells as "non-self" in presence of major histocompatibility complex (HC) determinants. Non-self antigens may represent HIV structures on cells replicating HIV or HIV-envelope-protein gp 120 attached on T_4-receptor of cells. Immune cascades via interaction of antigen presenting cells (APC), e.g. monocytes, with T_4-helper/inducer cells (T_H) are induced. Release of cytokines including interferon gamma (IFN-γ) and destruction of infected T_4-cells by T_C is initiated. Neopterin levels are increased in patients.

Fig. 1

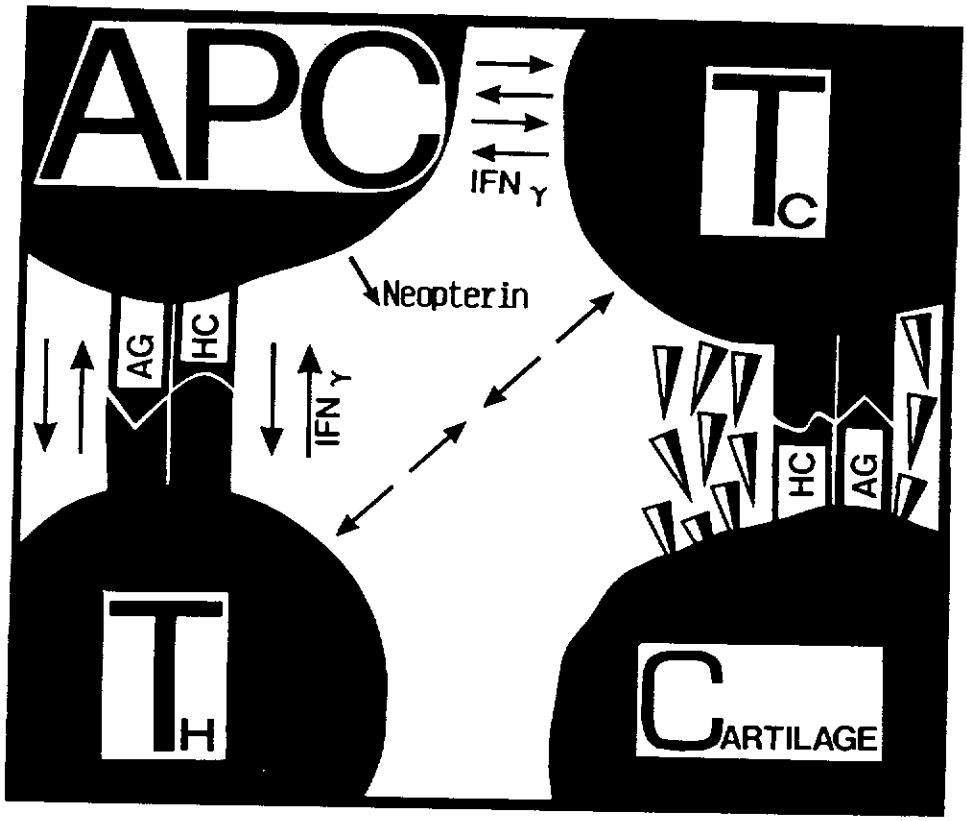


Fig. 2

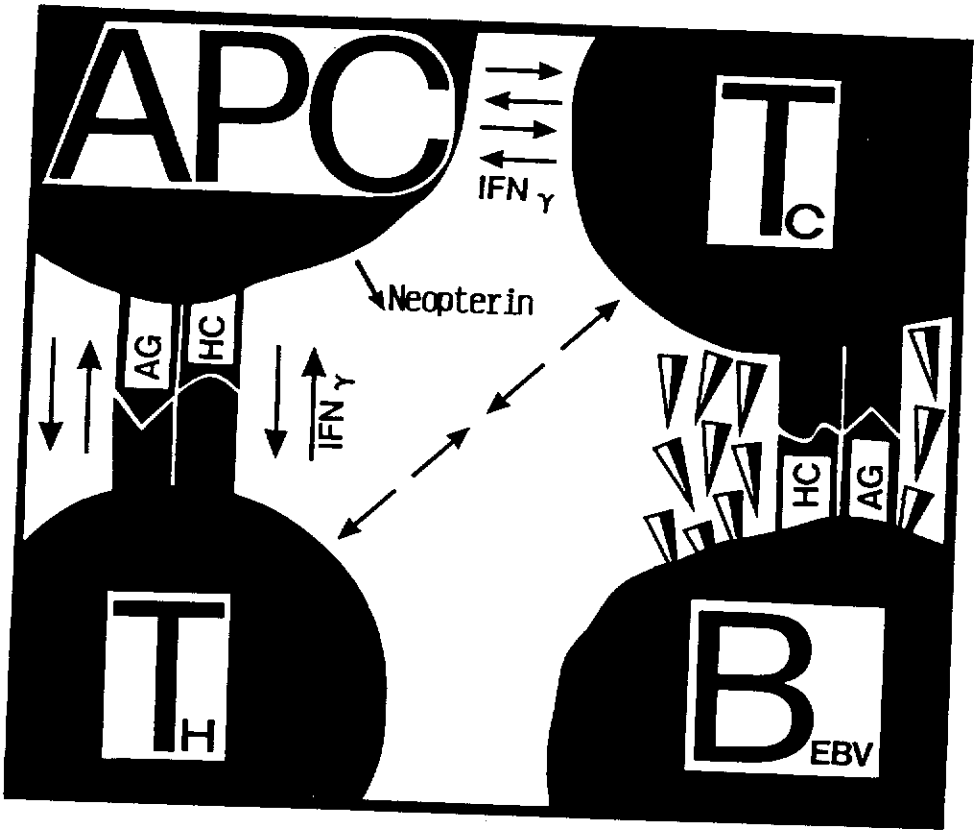
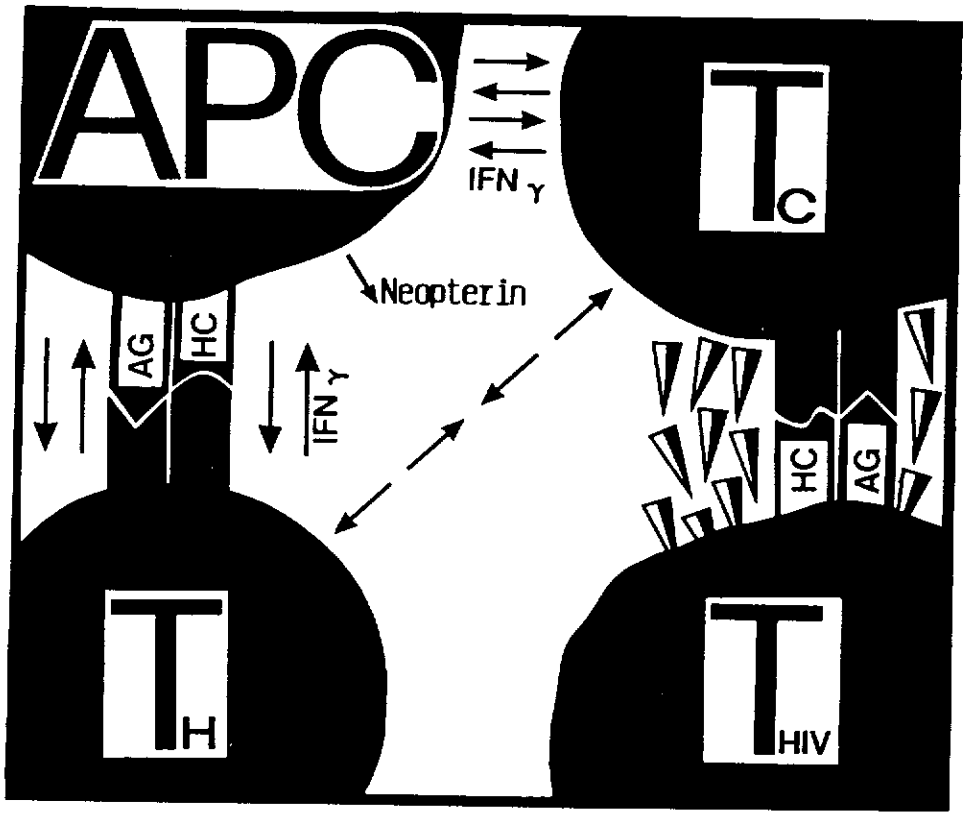


Fig. 3



PHARMACOLOGY OF ANTIVIRAL DRUGS

Donald Armstrong, M.D.

The only proven effective treatment for human immunodeficiency virus (HIV) infection is zidovudine, a nucleoside analogue. However, before HIV was identified as the etiologic agent of AIDS, alpha interferon produced clinical responses in some patients with Kaposi's sarcoma; the mechanism underlying this response remains unclear. Zidovudine has been shown to prolong life and decrease the severity and number of opportunistic infections in a subset of patients with AIDS. Indeed, the most promising compounds in preclinical evaluation to date have belonged to the nucleoside analogue class.

Agents other than nucleoside analogues with activity against HIV have been identified; however, the role for most of these in the treatment of HIV infection and AIDS has yet to be defined. There are, however, a few statements that may be made with relative certainty. Alpha-interferon has clearly found its niche in the treatment of Kaposi's sarcoma and may be of value when used in combination with an antiretroviral agent. Treatment with suramin, the first anti-HIV agent studied, has not been associated with clinical, virologic, or immunologic improvement and is highly toxic; no further studies of suramin in HIV-infected individuals are planned.

For some of the agents discussed, such as AL-721 and peptide T, reproducible evidence of anti-HIV activity <u>in vitro</u> is lacking and should be obtained prior to the initiation of clinical studies. And as underscored by the experience with suramin, activity <u>in vitro</u> does not necessarily translate into clinical efficacy.

The evidence for synergistic inhibition of HIV <u>in vitro</u> is encouraging and paves the way for clinical study of combination chemotherapy for HIV infection. This approach has been used successfully for the treatment of neoplastic disease and some infectious diseases. Such studies are currently in progress.

As with many viral infections, the natural history of HIV infection is variable. Consequently, anecdotal reports of efficacy are difficult to evaluate. For this reason, carefully controlled trials of new agents, first evaluating safety and pharmacokinetics (phase I), and then efficacy (phase II), will be required. Studies of efficacy should be multicenter collaborative trials, usually randomized and double-blind, and new agents should ultimately be tested against the established therapy of the day. In the United States, the NIH-sponsored National Cooperative Drug Discovery Groups for the Treatment of AIDS (NCDDG-AIDS) and the ATEUs provide a mechanism for the development and testing of new treatments for HIV infection and AIDS. We are optimistic that these and other collaborative research efforts will contribute to the successful control of this epidemic.

VENDREDI 8 JUILLET 1988

14 h 30 - 18 h

STRUCTURE ET FONCTION DES PROTEINES MEMBRANAIRES

MEMBRANE PROTEINS STRUCTURE AND FUNCTIONS

The Flagellar Rotary Motor

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Many bacteria swim by rotating thin helical filaments that extend from the surface of the cell out into the surrounding aqueous medium. Each filament is driven at its base by a reversible rotary motor, whose specifications (in plain English) are summarized below:

Feature	Specifications
Diameter	1 micro inch
Speed	6,000 rpm
Power output	1/10 micro micro micro hp
Power output per unit weight	10 hp per pound
Power source	Proton current
Cylinders	8
Number of different kinds of parts	30
Gears	Forward and reverse

For one guess as to how this motor might work -- I will tell you more about this at the Conference -- see Berg and Khan (1983) or Khan and Berg (1983). The speed is the value recorded in swimming cells at room temperature (Lowe *et al.*, 1987). The power output seems miniscule, until one divides by the weight: internal combustion engines do no better (cf. McMahon and Bonner, 1983). The motor is driven by an inward flux of protons (or hydronium ions); about 1000 are required per revolution (Meister *et al.*, 1987). Proceeding more systematically than possible in earlier work (Block and Berg, 1984), David Blair has induced the synthesis of wild-type MotA and/or MotB in *mot*-minus strains of *Escherichia coli* and found several examples in which cells tethered to

glass by a single flagellar filament reach their top speed in eight steps, so we now believe that there are only eight independent force-generating units (Blair, D.F., and H.C. Berg, in preparation). It is the gears that are critical for sensory transduction, (e.g. chemotaxis).

When a cell such as *E. coli* swims, it turns its motors alternately clockwise and counterclockwise. Counterclockwise rotation enables it to swim smoothly along a gently curved path (to run). Clockwise rotation enables it to move erratically with little net displacement (to tumble), and hence to run again in a new direction chosen approximately at random. By biasing this random walk (e.g., by extending runs that carry it up a gradient of a chemical attractant) the cell can migrate in directions that it finds more favorable. For a brief review of bacterial chemotaxis, see Berg (1988).

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NEW SPIN LABELLING APPROACHES TO THE STUDY OF PROTEIN STRUCTURE AND FUNCTION IN MEMBRANES.

Wayne L. Hubbell*, Paul Todd*, Veronica Crozel+, Francoise Levinthal+ and Cyrus Levinthal+

I. Introduction The structure and function of membrane proteins is a rich area of investigation which promises to yield new conceptual advances in biophysics. Many of the proteins of interest are too rare or too large for structural studies by nuclear resonance and have little hope of direct structure determination by crystallographic methods in the near future due to their rarity or difficulty of crystallization. Important classes of such proteins are voltage- and chemically-gated ion channels of neuronal origin, energy-linked transport proteins and receptor proteins. These are the cases in which molecular "probe" techniques have a particular advantage for study of structure and function. The nitroxide spin labelling technique is arguably the most general of such approaches in terms of the information obtained (For a complete overview of the technique, see ref. 1). In this method, a nitroxide group such as that shown in Figure 1 is attached covalently or non-covalently to a specific site in the structure of interest by virtue of a reactive functionality designated as "R".

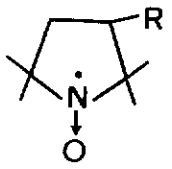

Figure 1

The Electron Paramagnetic Resonance (EPR) spectrum of the attached stable free radical provides a wealth of information on the properties of the local environment and time dependent changes in these properties. The spectra are sensitive to molecular motions from 10^{-12} to 10^{-3} seconds, to dielectric constants in the range of 2 to 80, to anisotropy of motion and net orientation relative to a laboratory frame. The very small size of the nitroxide functionality produces minimal perturbation in the structure of the system.

Spin labelling has proved to be an extremely successful approach for the investigation of lipid molecular dynamics in membrane systems. For example, the discovery of the fluidity gradient in the hydrocarbon chain region of the phospholipid bilayer (2,3), the rate of phospholipid "flip-flop" across the bilayer (4), the translational diffusion coefficient of phospholipids (5,6), and the existence of boundary layer lipid around membrane proteins (7) were all investigated quantitatively the first time with spin labelled lipids. The spin label technique has enjoyed less success in the study of membrane proteins, although in principle the type of information provided is ideally suited to the nature of the problems of interest. The reason for this is twofold. First, the sensitivity of the method for limited aqueous samples has been rather low, even though it is intrinsically~1000 times more sensitive than NMR. Secondly, and most importantly, it has been exceedingly difficult to selectively place covalently bound nitroxide groups in proteins which generally have many chemically reactive sites.

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Recent developments in microwave technology and molecular genetics have solved these problems for some important cases and opened the door to the application of this powerful technique. The significance of these advances and a specific application are discussed briefly below.

II. Advances in Microwave Technology Conventional cavity resonators used to detect the resonance condition in EPR spectroscopy are high Q, low filling-factor devices when used with aqueous samples. Recently, Froncisz and Hyde introduced a new resonator to the EPR field, the loop-gap resonator (LGR) (8). The LGR is a low Q, high filling factor device which has improved sensitivity at least two orders of magnitude (three order of magnitude for some experiments) for limited samples in aqueous systems. In addition, the low Q feature of the LGR has made practical the application of double resonance EPR (ELDOR) and time domain EPR (saturation recovery) to limited samples, thus providing entirely new dimensions to the information obtainable (9-11). We have found it possible to routinely work with 50 picomoles of spin labelled protein, which brings the spin labelling method into the range of sensitivity required for the study of many rare proteins.

III. Genetically Engineered Labeling Sites The difficulty in achieving specific site labelling has been a major deterrent in applying spin labelling to proteins. However, the recent development of site-directed mutagenesis has made it possible to engineer specific sites in a protein sequence that have a unique chemistry to which a spin label may be covalently attached. To the extent that the mutants can be prepared, this approach completely solves the problem of selectivity. A very convenient and general labelling site is provided by a sulfhydryl group, and the systematic replacement of single amino acids by cysteine can provide a series of mutants ideally suited to the labelling approach. Non-essential native cysteines can be replaced by non-reactive residues to ensure unique labelling sites. Point mutations of the type proposed are anticipated to produce only localized structural perturbations and to preserve global structure and function. The validity of this assertion is currently being investigated (see below).

Mutant proteins, especially those of mammalian origin, are often available in very small amounts. Thus the combined application of both LGR and genetic engineering technology promises to revolutionize the application of spin labelling EPR to protein systems.

IV. Applications to the Study of Colicin E1, a Channel Forming Membrane Protein.

Colicin E1 is a cytotoxic protein of 56,000 MW produced by the bacterium E. Coli(12). The protein is synthesized in a water soluble conformation, but upon encountering a membrane interface it spontaneous inserts into the structure to become a voltage-dependent ion channel. It has recently been shown that only a single polypeptide domain consisting of the C-terminal 88 amino acids is required for channel formation (13). It is thus an extremely attractive simple model for both protein insertion into membranes and voltage-dependent channel formation.

So far 20 mutants in the C terminal half of colicin E1 differing only by the position of a single cysteine in the sequence have been produced. These are at positions 340, 398-406, 482, 483, 485, 487-492, and 520. Each of these mutants has been selectively labelled by nitroxide spin labels in amounts sufficient to obtain EPR spectra of excellent signal-to-noise. The spectrum of each labelled mutant is unique and has provided important information regarding the environment and structure in the immediate vicinity of the labelled site. Together, the set of labelled mutants provides a more global view of the protein organization. For example in the water-soluble form of the protein it appears that residues 399-406 are part of an amphiphilic alpha-helical sequence. This conclusion is based on the known sequence and an obvious periodicity in the EPR spectral properties which reflect periodic alternations of both mobility and polarity. One face of this putative helical segment faces the aqueous medium and the other the interior of the protein(14).

The sequence 482-492 is extremely hydrophobic, and accordingly the EPR spectra of the labelled mutants suggest that this sequence is buried within the protein interior, completely protected from contact with water.

The binding of colicin to the membrane is a process of great interest. The spin labelled mutants have made it possible to study in a direct way both the kinetics of the binding and certain aspects of the structure of the final equilibrium state. When colicin binds to the membrane, the spectra of most of the labeled mutants undergo dramatic changes which suggest that the polar surface of the amphiphilic helix in the 399-406 region interacts directly with the membrane surface, while the hydrophobic sequence 482-492 is inserted into the low dielectric membrane interior.

The spectral changes observed upon membrane binding make it possible to study the kinetics of membrane insertion with respect to each labelled domain. Studies to date have shown that the kinetics of membrane association and insertion are highly pH and salt-dependent for interaction with charged bilayers. Under conditions where insertion is rapid for charged membranes, insertion into neutral membranes is very slow although the same equilibrium state is finally reached. These results suggests that the local charge distribution on the protein may be of importance in determining the correct orientation of the protein for the initial adsorption event.

Studies currently underway make use of the labelled mutants in the hydrophobic C-terminal domain to determine the structure of the open channel state. Successful completion of these experiments requires a method for producing controlled and persistent transmembrane potentials in small phospholipid vesicles containing the protein. Several promising approaches are under investigation.

Obvious extensions of the present approach will involve producing mutants with two labelled sites. Through-space dipolar interactions between radicals attached at the two sites will be used to determine the inter-radical distance. Estimation of a few critical distances between known positions in the sequence will provide strict constraints on proposed structures.

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INTRAMOLECULAR PROTON TRANSDUCTION IN BACTERIORHODOPSIN

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Bacteriorhodopsin, one of four retinal-containing proteins of Halobacterium halobium, generates upon light excitation an electrochemical gradient based upon a delicate intramolecular mechanism of retinal isomerization coupled to deprotonation-protonation cycles, as follows:

1. A light-induced quasi-irreversible isomerization of the protonated Schiff-base of an all-trans retinal chromophore yields a deprotonated Schiff-base of the 13cis, 14s-cis-retinal isomer.
2. The isomerization triggers via a deprotonation of a protonated aspartate residue in the neighbourhood of the Schiff-base a protonation transfer reaction cycle toward the outside of the membrane.
3. These reactions are coordinated in time with reprotonation events occuring from the inside of the membrane toward the active site being a prerequisite for the reisomerization process yielding the all-trans ground state.
4. The control function of a cation of the reisomerization reaction as well as the ground state was only recently recognized.

The mechanism is based on our konwledge of the primary and secondary structure of bacteriorhodopsin and essentially on results obtained by NMR-spectroscopy, Raman spectroscopy, by static and time-resolved infrared difference spectroscopy (including FTIR).
For IR experiments see:

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BUNDLES OF HYDROPHOBIC α -HELICES AND TRANSMEMBRANE CHANNELS.

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One of the most favored hypotheses concerning the ionic channels formed by transmembrane proteins, is that "hydrophobic" segments contained in their sequence are inserted in the membrane as α -helices assembled in "bundles" enclosing a pore apt to transport ions.

In the absence of sufficient crystal data on intrinsic membrane proteins and in view of contributing to the understanding of the possibilities of formation and of the functionning of such bundles as channels a theoretical study was undertaken on the aggregation properties of hydrophobic α -helices and of their capacity to function as channels [1-5].

Among the most important results are :

1) the determination of the relative importance of the electrostatic and dispersion (London) components of the energy in the interaction of a pair of hydrophobic α -helices [1] : while the electrostatic component tends towards an asymptot when the length of the helix increases, the attractive dispersion term due to the hydrophobic residues increases continuously. As a result, a parallel pair of essentially hydrophobic helices of a length sufficient to span a membrane is stable. For the same reason a "bundle" of such helices containing one or more parallel pairs can also be stable. Hence, it appears that the hydrophobic amino acids not only induce a favorable interaction of the helices with the lipid phase, but also insure a favorable interaction between the helices within the membranes.

2) Energy-optimization of aggregates of N-hydrophobic α-helices showed [2] that for each N, different bundles can be found, differing somewhat in shape, but with very similar stabilities, suggesting the possibility of transformation, from one to another by relatively small displacements within the aggregate, involving for instance, sliding and/or tilting of the helices. Such transformations could possibly be involved in the opening or closing of channels [4]

3) Some of the bundles formed were shown explicitely to be able to enclose a pore in their interior, capable to accomodate an ion [2,4]

4) The calculation of the energy profile of a sodium ion in the "channel" formed inside a bundle of five α -helices made of purely hydrocarbon amino acids showed that the interaction energy is favorable all the way through, due to the favorable interaction of the ion with the carbonyl oxygens lining the channel inner wall, and in spite of the absence of internal polar residues.

5) Replacement of the inner hydrocarbon residues by polar (non ionized) ones, for instance serines, leads to a deepening of the energy profile due to the favorable interaction of the ion with the polar head of the side chain.

These side chains utilize the flexibility of their hydrocarbon part to turn their attractive atoms towards the ion so as to ensure the best possible interaction at every height until "passing it over" to the next available group[3]. The lining up of ionic residues on the inner wall is not necessary for ion transport.

6) Extending these methodologies to the study of the functionning of the acetylcholine receptor channel, utilizing the exact amino acid sequences of the M2 helices of the five subunits presently considered to line the channel shows explicitly [6] :

a) that the blocking of chlorpromazine at the level of the labelled serines suggests strongly a direct contact between the M2 segments of the sub-units in this part of the channel.

b) that within this hypothesis, the channel can accommodate the largest of the cations known to be transported : dimethylethylammonium.

c) that the exit of the ion is made possible by the presence of the ionized carboxylates at the lower extremity of the channel.

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Molecular Dynamics Simulation of Primary Processes of the Photosynthetic Reaction Center of *Rhodospeudomonas viridis*

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The photosynthetic reaction center of *Rh. vir.*, a membrane protein (complex), transforms light energy into an electrical membrane potential by means of light-induced vectorial electron and proton transfer steps. We have investigated in how far the dynamics and the electrostatic forces in the reaction center interior contribute to the high efficiency of energy conversion. We identified an extremely fast and energy efficient relaxation step, correlated thermal motions involving constituents of the electron pathway, a proton channel to the cytoplasmic side, an effect of the inhomogeneous dielectric environment (membrane and aqueous phase) on the electrostatics inside the reaction center, and an effect of site-specific modification on global protein structure and energy conversion efficiency.

IS THE BASIS OF LIFE CHEMISTRY OR INTELLIGENCE?

The idea that intelligence underlies the phenomenon of life is one that is not, at the present time, a popular one in the scientific world. Niels Bohr's thoughtful arguments against quantum mechanics being able to give a fully adequate description of life have been widely ignored and have faded into the mists of the past, and the view that prevails instead is that a basic understanding of the phenomenon of life has already been achieved, so that all that remains to do now in biology is essentially the task of filling in more and more matters of detail. In fact this is an illusory view: as can be demonstrated by the consideration of various analogous situations, just knowing the chemistry of living organisms and their functional organisation, even in great detail, is very different from being in a situation where it can be said that the phenomenon of life has been essentially explained by science.

Artificial intelligence research has shown that intelligence has universal characteristics that can be studied objectively. Can one put this idea of a fundamental intelligence underlying life and other phenomena on to a proper scientific basis? Analysis of two recently proposed concepts, those of the fundamentality of meaning that has discussed by David Bohm, and of the self-referentiality of nature at its deepest levels that has described by Maharishi Mahesh Yogi, indicates how this may be possible.

DNA has the self-referential property of acting as a code that prescribes (among other things) the particular mechanism in the organism that gives the code its meaning. However, the DNA has its significance at the level of chemistry only; compared with ordinary language its capacity for acting as an agent of organisation and intelligence is extremely limited. Thus an appropriate basis for the proposed hypothesis of a fundamental underlying intelligence is the assumption that, at the finest levels, the structure of reality is one that in its general features mirrors that of the life process as conventionally understood, except that in it the codes that correspond to the DNA and RNA in the life process function with the dynamics and the semantics of the thinking process and of intelligence instead, in a way that is partly comprehensible in terms of the concepts that have been developed in artificial intelligence and in the other cognitive sciences. This concept can be seen to make contact with orthodox science in a number of ways.

The idea that the phenomenon of life does not depend in any way on a deeper-level organising process is one that fits in well with a materialistic world view. But it is one that I suspect will, in the end, be seen to be false.