assumed by Kimura. Alternatively, if $m < ur$, N_e is the appropriate population size, but in this case hybrids between populations would be heterozygous at almost all loci, and would be polymorphic for different alleles at any given locus. There is evidence (Stone *et al.*, 1968) that for Drosophila on pacific islands, this is not the case. In fact, if $m < ur$ for a period long enough to affect the equilibrium described by equation (1), we should probably describe the different populations as separate species.

It seems therefore that the agreement with equation (1) obtained by Kimura for Drosophila is spurious, and that if neutral mutation is as common as he supposes then a far larger proportion of loci should be heterozygous in wild populations than is in fact the case. Unfortunately, this discrepancy is insufficient to disprove the neutral mutation hypothesis, since it may be that Kimura has seriously overestimated the neutral mutation rate. The difficulty arises because in Drosophila we cannot estimate u from the rate of gene substitution, as might be possible in the case of mammals, since no proteins have been sequenced.

Equation (1) should however provide a way of testing the neutral mutation hypothesis. For example, comparing different species of mammals, one would expect a far higher degree of genetic polymorphism in, for example, a rodent with a continental distribution than in a large ungulate confined to a small geographical range.

There is however a serious difficulty in using equation (1) to test the drift hypothesis. This is that it describes an equilibrium which is reached very slowly. The number of generations required to approach the equilibrium. (e.g. after a disturbance due to a change in N_e) is of the same order of magnitude as N_e; hence no large population will be anywhere near its equilibrium. This is particularly serious when considering a species such as our own, which has increased by a factor of perhaps 10^4 in the last 400 generations. Nevertheless, there may be a way of testing the neutral mutation theory from data on the frequencies of variant proteins in man.

5. Gene frequency distributions in man

The approach to an equilibrium between mutation and elimination is exceedingly slow in a large population. Hence if we want to test the neutral mutation theory by comparing observed and theoretical gene frequency distributions, we need to work out what the gene frequency will be in a population changing in size. It turns out that this may be possible for a population such as our own which has increased by several orders of magnitude in the recent past.

Every gene at a particular locus in the present population is a copy, without mutation, of a gene which arose by mutation some specific number n generations in the past. Let F_n be the probability that a gene, chosen at

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random from the present population, arose exactly n generations ago. Let N_0 and N_n be the population size now, and n generations in the past respectively.

The number of new selectively neutral mutations arising exactly n generations ago was $2\,N_n u$. If, as we must on the drift hypothesis, we ignore selectively advantageous mutation, the expected number of copies now of every gene (including new mutations) present n generations ago is $N_0\,(1 - P_{n-1})/N_n$, where P_{n-1} is the fraction of genes now which are copies of genes arising by mutation during the last $(n - 1)$ generations. Hence if $P_{n-1} \ll 1$, we have $\mathrm{F}_n = 2\,N_n u \times \mathrm{N}_0/N_n \div 2\,N_0 = u$ *.

Thus the neutral mutation theory leads to the very simple conclusion that, provided $nu \ll 1$, the fraction of genes now present which arose by mutation during the period from r to n generations ago is $(n - r)\,u$. This can form the basis for a frequency distribution of variant alleles in the present population.

The derivation of frequency distributions is now being undertaken. However, it already seems likely that the frequency of rare haemoglobins in man will not agree well with the frequencies predicted from the neutral mutation theory. Considering the observational data first, Lehmann (quoted in Harris, 1970) in a sample of 10971 individuals from Western Europe found 10 rare electrophoretically separable haemoglobin variants, 3 in the α and 7 in the β chain. Of the β chain variants, 3 are common in other parts of the world and thought to be maintained by heterozygous advantage. Of the remaining 7 variants, 4 occurred once and 3 twice. Hence all had frequencies less than 10^{-4}. Together, these very rare variants contribute a fraction of approximately 2.5×10^{-4} of the genes at each of the α and β loci.

An idea of the distribution to be expected on the neutral mutation theory can be obtained as follows. Let the probability that a new mutation arising n generations ago is still represented in the population be P_n. For all mutations occurring n generations ago the expected number of copies now is N_0/N_n, and hence for each surviving mutation the expected number of copies is N_0/N_nP_n, and hence its expected frequency now is $1/N_nP_n$. For $n > 400$ the human population was probably small - perhaps of the order of 10^5. P_n for $n > 400$ will be of the order of 10^{-2} or less. Hence alleles arising by mutation 400 generations or more in the past will, if still present, have frequencies of 10^{-3} or greater. No such alleles were found in Lehmann's samples (if we discount the alleles thought to be maintained by heterozygous advantage), although if the period from 0 to 400 generations ago has provided a fraction of 2.5×10^{-4} of existing genes, we would expect that the period from 400 to 4000 generations should provide 2.5×10^{-3} of existing genes, or perhaps one or two alleles with frequencies of 10^{-3} at each of the α and β loci. We would also expect some alleles with still higher frequencies originating still further in the past.

* The precise expression for F_n, when P_{n-1} is not very small, is $F_n = u\,e^{-un}$.

The objection might be raised that it is inappropriate to consider electrophoretically separable variants as typical of selectively neutral variants. But in the evolutionary divergence of the α and β chains, there have been 60 substitutions which do not involve a charge change and 24 which do, corresponding roughly to the ratio to be expected if the two classes are equally likely to be selectively neutral.

The simplest explanation of the discrepancy is that selectively neutral mutations do not occur. The very rare variants are slightly disadvantageous, but have not yet had time to be eliminated. Variants arising more than 400 generations ago have been eliminated.

There is however one other possible explanation, consistent with retaining the view that most gene substitutions in evolution are selectively neutral. Suppose that our species has in the comparatively recent past (say 5000 generations, or 100,000 years) gone through a " bottleneck" of very small numbers, leading to genetic homozygosity at many loci, including haemoglobin. On this view, the absence of variant haemoglobins with frequencies of 10^{-3} or greater is explained by inbreeding *. This view would be consistent with the existence of other loci in man in which two or a small number of selectively neutral alleles were common, since the degree of inbreeding might have been sufficient to produce homozygosity at some but not all loci. But it would be inconsistent with the presence of loci at which an appreciable number of alleles have frequencies of 10^{-3} or more.

If there has been such a bottleneck, it should be possible to estimate approximately how small the population must have been. The bottleneck must have occurred in the last 100,000 years, since if it had occurred earlier we would expect to detect more variants which have arisen since that time.

More work, both in collecting data on protein variants and in deriving the theoretical distributions, is needed before drawing any firm conclusions. Perhaps the most interesting possibility is this. If we are obliged by other evidence to accept the neutral mutation theory, then we may be able to obtain an idea of the size of populations ancestral to our own from a study of protein variation in existing populations.

* The idea that close inbreeding would lead to an absence of variant proteins is not inconsistent with the equation $F_n = u\,e^{-un}$. In this equation, F_n is the *mean* fraction of genes in the present population which originated n generations ago. If the human population was closely inbred in the past, the variance of F_n would be very large. Thus imagine a large number of populations each having the same past history of numbers as our own. Then if this past history involved recent close inbreeding, F_n gives (approximately) the probability that a particular population is homozygous for a gene originating n generations ago. But if there has been no recent inbreeding, F_n will approximate to the fraction of each population consisting of such genes.

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DISCUSSIONS

C. LEVINTHAL : What is the relationship between Lewontin's experiments and the lack of variation you discuss ?

J. MAYNARD SMITH : Lewontin was one of the first population geneticists to exploit protein variation. He is one of the people responsible for my dogmatic statement that populations are a great deal more variable than we had thought. When he first found this, he thought it must be explained by neutral mutation, because the extent of it is too great to be accounted for by natural selection. He said this because he, as we all did at that time, accepted Haldane's cost of selection argument. However I think Lewontin would now accept that this protein variation could be explained either way. Since that time, Lewontin and others have studied protein variation in flies from different local populations of the same species — for example on different islands. If you have a population divided up into small groups and it turns out that each group has the same variant, then this is very difficult to explain on a random basis. It suggests that each group must be stable, otherwise they wouldn't be the same, they would drift apart. A lot of work of this kind is going on at that moment. It's difficult to interpret precisely because of the difficulty that the equilibrium is approached very slowly. Unless you know quite a lot about the past numbers of the animal you are looking at, it's difficult to interpret the data. I have a feeling that our own species, for which we have more idea about past population numbers than for the fruit fly, may be a better bet than fruit flies because of this particular problem.

H. FRÖHLICH : Is there anything known about the physical processes that lead to a mutation ?

D. GLASER : I have been asked to answer this, which I will do by expressing, the prevailing prejudice that, except for occasional hot spots, the probability of a base change is the same everywhere in the chromosome. Whether a base change is reflected in an amino-acid change which can have a phenotypic effect, depends on the considerations that Pr. Margoliash outlined for us. Since leucine is represented by four possible base triplets and methionine by only one, a particular protein rich in leucine, can tolerate more base changes without suffering a change in the amino-acid leucine, then if it is rich in

methionine. In general, I think, the prevailing prejudice is that the probability of a base change is uniform along the DNA, but doesn't produce uniform consequences in the amino-acids. And then it's a very open question which Pr. Margoliash discussed at length, whether amino acid changes produce phenotypic changes. But maybe we can get a professional answer.

D. GLASER : I have a question : is it really necessary that we decide between the two competing theories. Isn't it more likely that there is a range of proteins, some of which can sustain large numbers of neutral mutations, and others of which, because of the sensitivity of their structure to their sequence or because of their critical importance to the organism, cannot tolerate the slightest change in their structure.

E. MARGOLIASH : It is important to point out something different. Lewontin's experiments are also based on electrophoresis of *Drosophila* extracts. Therefore, the estimate of heterogeneity obtained is a minimal one, since not all changes of structure necessarily produce an electrophoretic variation. We may in fact be dealing with much more than he actually observed.

J. MAYNARD SMITH : I think you are right that we don't have to take a black or white choice here. However I should perhaps explain that in the history of population genetics almost religious feelings were aroused by the problem of whether anything could be selectively neutral. There are men, you know, who will go to the length of saying that nothing can possibly be selectively neutral. So for such people, once you admit that any mutation is selectively neutral, you have really sold out to the devil. Now I don't take this view. I think it perfectly possible to say that some proteins, say fibrinopeptide, can vary a lot without mattering, others are very tightly constrained. But in Dr. Margoliash's study of cytochrome c., it will probably turn out that one of two things is true. Either the great majority of the variations are selectively neutral, or the great majority have a selective interpretation which for the moment we cannot see. I'd be surprised if it turned out that 50 % was of one class, and 50 % was of the other. But that's just hunch.

J. BRONOWSKI : I want to ask a question about this last argument which is particularly interesting because it is quite new. Are not the calculations going to be seriously affected by whatever assumptions you make as to when perfect mixing began to take place ? In other words, how many highly homogenous human populations which were separated 500 generations ago have entered the stream that you are measuring now ? And, of course, the analysis also makes assumptions about selective mating which run all through the argument.

J. MAYNARD SMITH : This is the real difficulty. However it may not be as severe a difficulty as at first one might think. I have been looking at the problem of how much interchange of genes there must be between populations in order, for the purpose of these calculations, for them all to be effectively one population. A degree of migration of the order of the mutation rate is all you need. So, unless one supposes total isolation, then I think probably the distortion won't be too big.

D. GLASER : I would like to make a comment about this calculation you say is in progress, and express pessimism that it will be useful. That process is not unlike the problem in bacterial genetics of counting the number of mutants in a population and trying to determine the mutation rate, as was done in the interpretation of the Luria-Delbrück experiment. The mathematics for that problem after many years of approximations has finally been worked out exactly by Benoit Mandelbrot and leads to a statistical formula in which enormous fluctuations are found in the final number of mutations. The fluctuations are so large that it is extremely difficult to give any kind of estimate about the mutation rate which one wants to determinate.

J. MAYNARD SMITH : I was worried by this as well. Of course you can calculate the means; it is calculating their variances which is difficult. But there is this difference. If we wanted to know how many mutations we expected to find in the existing human population which originated exactly 500 generations ago, it is fairly easy to estimate the mean. But its variance might be so big that it would mean nothing. This is essentially the problem in the Luria-Delbrück situation. We do not have this problem because I'm not interested in how many mutations originated exactly 500 generations ago. What I'm interested in is how many originated between 500 and say, 5 000 generations ago. Then I think the variances are going to shrink fairly fast. However the variance might remain large if the human population has been through a bottleneck of small numbers of the order of 100.

D. GLASER : In Mandelbrot's analysis, the statistical distributions are of the Pareto type, $P(x) \propto x^{-\alpha}$, $0 < \alpha \leqslant 2$, and do not possess mean values, but only percentiles.

G.M. EDELMAN : In your last example, wouldn't recombination play a big role if there were selection for heterozygosis in the population. I seem to remember that Wills and Lewontin have talked about this possibility. Wouldn't it tend to increase the amount of variation and possibly account for these results ?

J. MAYNARD SMITH : Yes indeed, but only if selection is involved, not if the mutations are neutral. The beauty of the neutral mutation theory is the mathe-

matics are much easier, so that we can work out the consequences of the neutral theory with adequate precision. We can then compare the predictions of the neutral theory with observation, and if that doesn't fit, then we know there's some selection, and that is the basic point we want to decide. If there is selection we shall have to start worrying about the point you raised about recombination, but on the whole population genetic calculations become dreadfully difficult as soon as you remember that genes are on chromosomes and are linked.

P.O. LÖWDIN : I will try to comment on Pr. Frölich's question concerning the physical reasons for mutations. The problem is not to give a series of possible mutation mechanisms, but to explain why mutations are so exceedingly rare in each one of them. If you look at the Watson-Crick model of DNA and the genetic code, it may be a good idea to start out with doubts and try to prove that the model is wrong or physically impossible — which is the starting point we chose in Uppsala in 1962 in our quantum-mechanical studies. The genetic code consists of a pattern of protons and electron-pairs forming hydrogen bonds, and, in each bond, the proton has two classical equilibrium positions corresponding to different information storage :

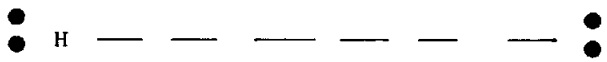

Fig. A

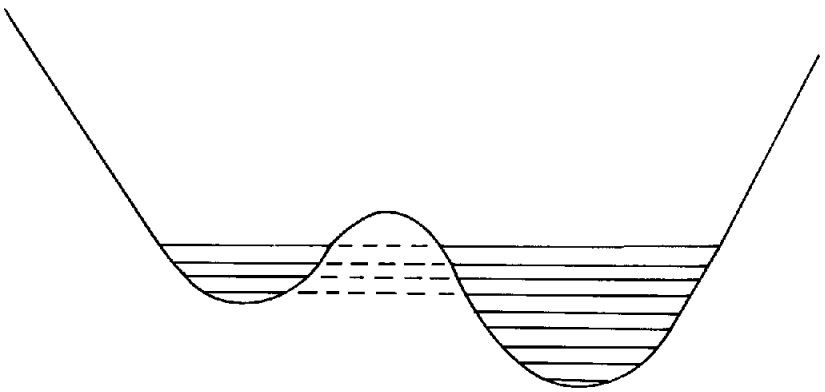

Fig. B

In quantum mechanics, the wave packet associated with the proton may " tunnel " between the two classical equilibrium positions, and the occupation of the tunneling levels depends on the temperature. At body temperature (310 °K), the tunnel effect corresponds to an " error " of 10^{-10} and 10^{-11} for the GC- and AT-base pairs, respectively, and these errors are associated with spontaneous mutation probabilities per base pair and generation. The

fact that these figures are so small indicate an essential stability of the genetic code in the Watson-Crick model; the corresponding experimental figures are estimated to lie between 10^{-8} and 10^{-12}. It is interesting that the AT-pair seems to be more stable than the GC-pair. If the nucleic acid is exposed to weak ultraviolet radiation, there are electron transfers in the base pair which may enhance the probability for a proton transfer by a factor 10^6.

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S. BENNETT : With reference to the question by Professor Bronowski, I wish to express my lack of understanding at the choice of 500 generations as fitting some significant point in human evolution. If I judge this correctly, this would be about the end of the Pleistocene and somewhere in the neighborhood of 15000 years ago. At that time there were many isolated human populations in many parts of North America and the Old World. To postulate some single genetic pool, or even a small bottleneck of some kind, strikes me as unreasonable. For this reason, I don't understand the merits of choosing this kind of a basis for the argument you present. It seems to me a much older, much longer series of generations would be necessary, if one is to draw on our present knowledge of human evolution.

J. MAYNARD SMITH : The choice is not based on our knowledge of human evolution in this case. The point is that comparatively recent mutations, let us say those occuring in the last 200 or 300 generations, would have very low frequencies in the present population. So that the known observation that there are a number of variants of haemoglobin with a very low frequency of 10^{-4} or less is consistant with the idea that these have originated in the last 300 generations. But if a mutation originated more than about 500 generations ago, and if such a mutation is still present in the population now, then it would probably have a frequency of more than 10^{-4}, and such variants have not been found.

K. MENDELSSOHN : May I just ask the chairman one thing : is there really a difference between the thermal mutation rate and mistakes in arrangement.

Are mistakes in arrangement necessarily part of the thermal or statistical variation under kT ?

D. GLASER : I'm sure that if you did a calculation you would probably identify several terms that contribute to the total rate. One term would be thermal dislocations in complete double helix structures; another would be thermal damage to a temporarily open singlestranded structure; a third one would be thermal disturbances in the fitting of the polymerase to the precursor and single-strand structure that it was working on; and probably another one would be a simply a quantum mechanical calculation of barrier penetration probabilites independent of kT. What I had in mind was the last one, when I spoke of quantum mechanical effects which were not thermal. I'm sure all these things would come together in determining the temperature dependence of mutation rates, for instance, but I imagine you have to estimate them separatly.

De la Physique théorique à la Biologie, C.N.R.S., 1971.

PHYSICAL AND CHEMICAL PROCESSES LEADING TO A MUTATION

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Mutation and selection are generally regarded as the main driving force of evolution. We have now a good classification of mutations : nonsense, missense, frame shift, deletions and chromosome aberrations are the main kinds, covering most of the events. A more complicated problem is how mutations are generated, what is the mechanism of the premutational act, how they become fixed and replicated in the progeny ? I shall concentrate on two different types of mutations — the spontaneous and induced ones. Both were studied in our laboratory using bacteria as a genetic model. What are spontaneous mutations ? Their probability is on the order of 10^{-7} - 10^{-9} for a specific gene per generation of cells. If we take for the number of acting genes 10^2 (the overall number of genes per bacterium is on the order of 10^3, but only a part of them is derepressed), so the number of mutations in any active gene would be 10^{-5} - 10^{-7} per generation. If we take 10^3 nucleotide pairs as an average dimension of a cistron it gives us the probability of replication mistake on the order of 10^{-8} - 10^{-10} per generation. We will take 10^{-9} as a mean. If this is an intrinsic probability of erroneous replication by DNA-polymerase during DNA synthesis we must consider a rate constant of the reaction of noncomplementary DNA replication. For bacteria the rate constant of regular DNA replication is on the order of 10^4 nucleotide pairs/second. Hence, for the rate constant of erroneous noncomplementary replication we obtain $10^{-9} \times 10^4$ nucleotide per/sec. $=$ 1 nucleotide per/day. We see that if we could stop the reaction of regular DNA replication but keep the cell alive and the DNA-polymerase active, we would obtain one wrong nucletide addition per day. This would be a premutational event. If the cell would grow afterwards and replicate its DNA in a normal way a mutant would result for every cell in an entire population. This experiment was really performed recently. We took thymine deficient cells of Bac. subtilis and E. coli and cultivated them in a medium with a very low concentration of thymine (0.2 - 0.3 μg/ml). It is a usual practice that bacterial cells die out in such conditions during 2 - 3 hours. The phenomenon of thymineless death was much studied last years and was regarded as a very general one. But we found that on a solid medium i.e. agar plates with a minimal medium supplemented with a very small amount

of thymine or devoid of thymine, thymineless death is practically absent. Cells can be washed off after 50 - 80 hours of thymine starvation and the cell count is constant. When a small thymine concentration is given, the cells even grow and divide. The surface of agar, where at first 10^4 - 10^5 cells were plated (on a Petri dish) becomes covered by microclones visible in the microscope. The cells are washed off afterwards and cultivated in a medium with 20 μg/ml of thymine and then tried for mutants. In conditions of thymine starvation an overall mutagenesis in the whole cell population develops. It starts after 20 hours, when the growth of the cell population comes to a stop. During 2 - 3 additional days the per cent of auxotrophic mutants increases and comes to 70 - 80 % of the whole cell population. Practically most of the cells are mutants and many of them multiple mutants. It is easy to observe the auxotrophic cells with deficiencies for aminoacids, nucleotides, vitamines. They do not grow on minimal agar or grow at a small rate (if the mutants are leaky). We identified some 10 - 12 of the mutants in different loci. We studied also revertants to prototrophy in tryp locus and drug resistant mutants (especially streptomycine resistant).

The frequency of any particular mutation was some 10^3 times higher in our conditions than in the control. (For instance the number of streptomycine resistant cells increased without selection till 10^{-2} % of the cell population, with less than 10^{-5} % in the control, growing without thymine starvation). Of course some of the mutations are lethal and this gives a small decrease of the cell population (some 30 - 40 %). It is obvious that we deal here with a phenomenon of complete mutagenesis in an entire cell population, mainly without death of the cells involved. We think that the only possible explanation is erroneous DNA replication. The scale of time for the occurence of mutations by replication is just what we estimated earlier. The frequency of erroneous replications is a function not only of DNA structure and the properties of nucleotides. It depends also on the enzyme specificity. This factor was studied by Yanofsky and others and special strains with mutated DNA-polymerase revealed increased probabilities of replication mistakes. This is obviously the explanation of special mutator genes- i.e. genetic loci, which imply to the organism and increased mutability and genetic instability.

Now we turn our attention to induced mutations, generated by some chemical substances or by UV-irradiation. We studied them by means of in vitro reaction with isolated and purified DNA with subsequent biological control of the DNA changes by means of transformation of bacteria (Bac. subtilis particularly). Of course we do not take here into consideration the mutations caused by aberrant DNA replication like those considered above. We can mention as example of the latter the increased mutability of bacteria with-5-bromouracil incorporated into their genome instead of thymine, probably because of tautomeric changes in the molecule, and also some specific muta-

genic poisons acting on DNA during replication (for instance acridine dyes). We shall concentrate on factors inducing stable chemical modifications in DNA and will consider the complicated sequence of events leading to a mutation by this mechanism. The chemical modification of DNA effected in vitro is the first event. Then we observe the uptake of DNA by competent cells, its integration into the genome and as a final result - new hereditory properties. All the agents studied by us (UV-light, nitrous acid, dimethylsulfate, nitrosonitromethylguanidine, hydroxylamine, hydrazine) cause mutations when acting on DNA in vitro. But with a probability 300 - 10,000times higher they causes nonspecific damages, which prevent the integration of damaged DNA into the chromosome. It was shown by us that the uptake of DNA by cells is decreased insignificantly by DNA modification. The crucial stage is apparently the synapsis of DNA with the recipient cell chromosome. Here the nonspecific hits are acting and their lack of specificity is obvious because the action of different inactivating agents is strictly additive. This allowed us to formulate a simple phenomenologic theory of DNA inactivation which was confirmed both in our laboratory and in many others. The inactivating hits are not integrated into the genome, but there occur, as mentioned earlier also some chemical events which are integrated and lead to mutations. Their probability is much inferior to that of inactivating hits. What are the premutational events in this case ? We could study them in case of UV-irradiation by means of the photoreactivating enzyme. The latter is well known to repair only pyrimidine dimers. We isolated this enzyme from yeast and acted upon UV-inactivated DNA before using it in transformation. We found that both inactivating damages and premutational damages are repaired at the same rate, i.e. are chemically equivalent. What is the difference then between the specific mutational damages and the nonspecific inactivating hits ? Probably this is a question of secondary DNA structure. The integrity of a straight DNA strand is important for its recombination ability. In particular crosslinks between both DNA strands and also dimerization with the formation of loops in one strand may be responsible for inactivating hits. If so, then only in case of dimerization of neighbouring pyrimidines we obtain a structure which is able to be inserted into the recipient genome. We shall not go into details of chemical interactions. The technique used enables us in principle to study the mechanism of premutational events and of nonspecific hits. The former and the latter are partly repaired in the recipient cell by dark repair enzymes. The use of special strains deficient in repair enzymes is a powerfull tool for the investigation of premutational DNA-modifications and their fate in the cell.

On the whole a study of the mechanism of mutagenesis both in the course of replication and chemically induced, gives us an insight into the details of evolutionary changes which took place on our planet at early times and proceed to act nowadays.

Journée du 3 juillet 1969

Deuxième séance

ALKALI ION CARRIERS : DYNAMICAL BEHAVIOUR

PRESIDENT D. GLASER

De la Physique théorique à la Biologie, C.N.R.S., 1971.

ALKALI ION CARRIERS : SPECIFICITY, ARCHITECTURE AND MECHANISMS

M. EIGEN and R. WINKLER

Göttingen, Germany

The " carrier " is an entity which facilitates a " dynamical " process, namely the transport of some molecule or ion through a membrane. An understanding of its functional mechanism requires studies of its *dynamical* behaviour.

In understanding the behaviour of alkali ions it may be of help to give a short survey of what is known about the dynamical properties of other ions in solution.

Figure 1 [1, 2] contains a summary of characteristic rates of substitution of water molecules from the inner coordination shell of various metal ions. This step turns out to be rate limiting for metal complex formation reactions. The presentation of such a " periodic table " of rate constants is meaningful

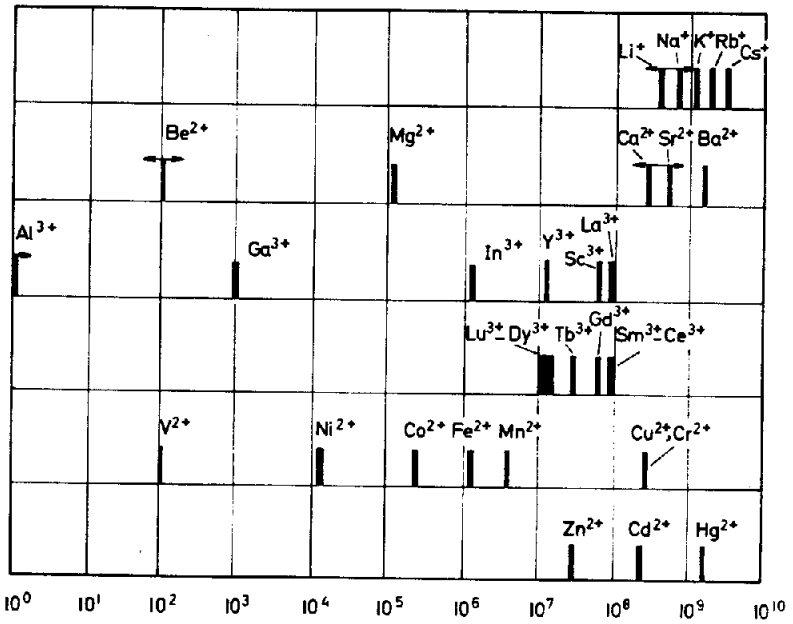


Fig. 1. Characteristic rate constants (sec^{-1}) for substitution of water molecules of the inner coordination sphere of various metal ions.

only, if the rates can be correlated to the properties (i.e. the electronic structure) of the metal ion only, independent of the nature of the incoming ligand. This indeed turned out to be so for most metal ions.

There are two facts to be taken from figure 1 which are of importance for the further discussion of alkali ion carriers: As will be seen this even holds for the formation of complexes where all the water molecules of the hydration sphere have to be substituted by ligands.

1. Any specificity in rates not following a simple metal ion radius dependence, can be found only with the transition metal ions [3], where typically chemical effects govern the substitution behaviour (as an example cf. the non monotonic radius dependence of substitution rates of transition metal ions as depicted in figure 2). In particular the well studied substitution processes

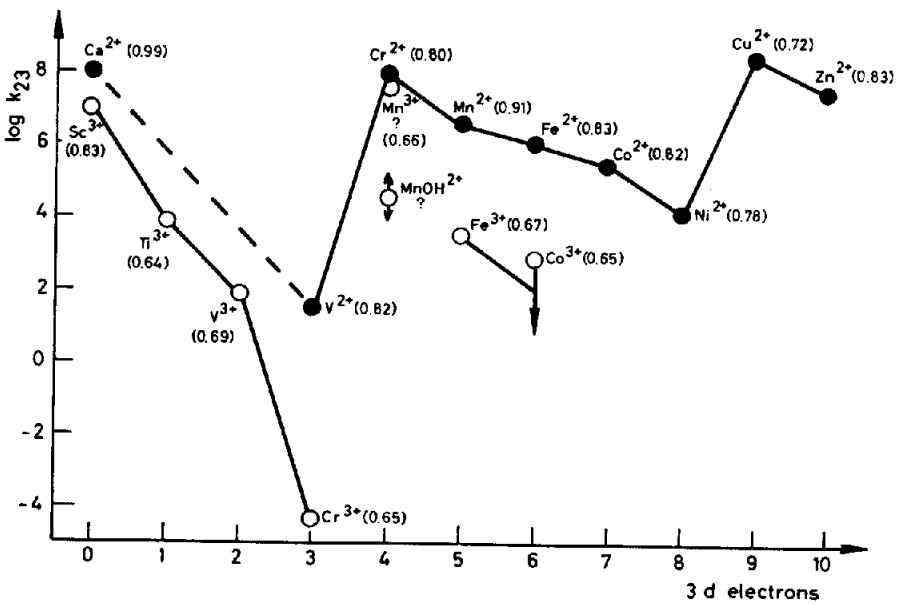


Fig. 2. Radius dependence of substitution rate constant (sec^{-1}) of transition metal ions.

for alkaline earth ions — irrespective of the ligand — show always high rate constants for Ca^{2+} and more than three orders of magnitude lower values for Mg^{2+} [4].

2. The alkali ions all are very fast in substituting single solvent molecules. The time constants are in the neighbourhood of 10^{-9} sec, with a slight radius dependence, i.e. increasing from Cs^{+} to Li^{+} [4].

Especially the second finding may be surprising if it is correlated with the well known solvation behaviour of alkali ions. Figure 3 shows the free energy of solvation for the different alkali ions as a function of the ionic

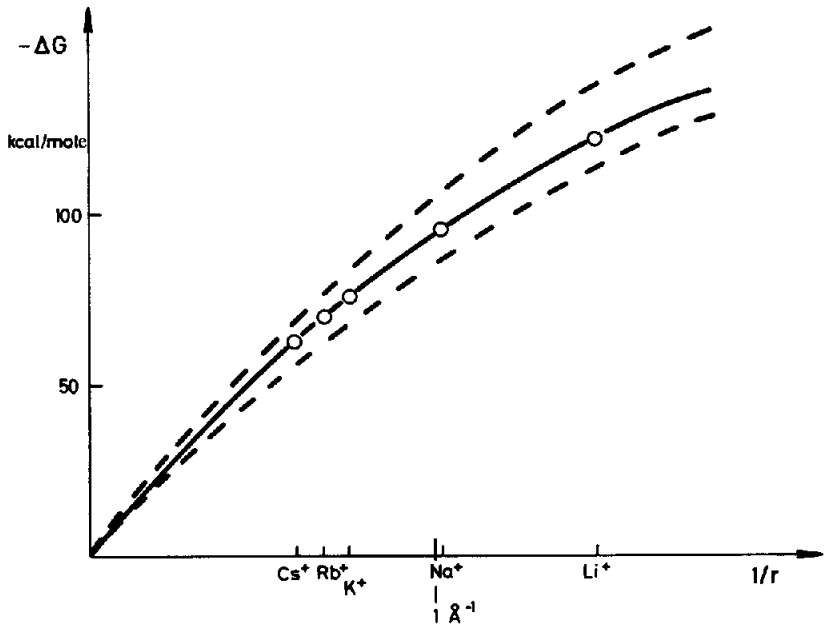


Fig. 3. Free energy of hypothetical ligand binding (dotted lines) and solvation (solid line, experimental values) as function of the reciprocal radius of alkali ions. (The ligand binding curve is related to a fixed ligand concentration.)

radius. The increase of the free energy of solvation with decreasing radius is monotonic as to be expected from any simple electrostatic picture. What is surprising is the relatively small correlation between solvation energy and rate. Despite the high solvation energy values, substitution is an extremely rapid process involving only a few kcal/mole of free energy of activation.

The two dotted curves in figure 3 indicate standardized free energies to be expected for complexes with ligands which are either more or less tightly bound than solvent molecules. No specific carrier behaviour can be deduced from such curves. The free energy of complex formation in the given solvent (here water) would be proportional to the differences between the broken and solid line. For simple ligands only monotonic behaviour, i.e. no maximum at any intermediate metal ion radius could be expected. Thus, the metal ion does not provide any specific property which would explain the specificity of a carrier. The metal ion specificity must be the consequence of a peculiar property of the carrier molecule utilizing the difference of solvation energy for the various alkali ions.

Figure 4 shows how such a behaviour can be envisaged. The two upper curves represent the free energy of binding for two different chelating agents. They consist of multidentate ligands which for complexation have to substitute

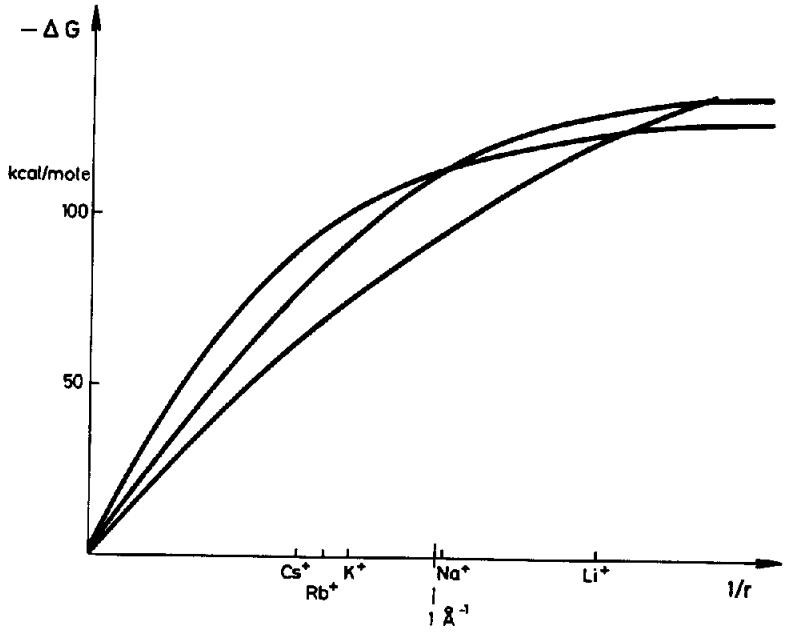
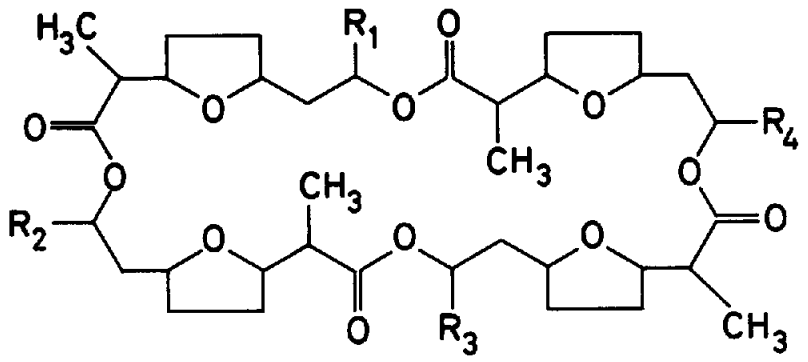


Fig. 4. Free energy of solvation (lower curve) and chelation (upper curve for two hypothetical cases) as function of the reciprocal radius of alkali ions.

the entire solvation sphere of the metal ion thereby enclosing the metal ion into a cavity. At large metal ion radius the free energy of interaction will increase monotonically with decreasing metal ion radius (even relative to the solvent if the ligand is favoured with respect to the solvent molecule as expressed by the higher interaction energy). A decrease of the metal ion radius is accompanied by a shrinkage of the size of the cavity. Due to steric hindrance and repulsion between the binding groups of the carrier the cavity soon will approach a minimum size, which will optimally fit a given metal ion radius. A further decrease of the metal ion size will then not result in any appreciable increase of binding energy (since the binding groups are " frozen " into fixed positions). Thus, the difference between ligand binding and solvation energy will pass through a maximum at a given size of the metal ion.

We have to conclude that binding specificity is a consequence of a specific architecture of the carrier utilizing the difference between free energy of solvation and ligand binding. This difference involves appreciable entropy increments in favour of the chelating ligand. A question remains : How can the process be facilitated quickly, since *total* desolvation is required which — even if one single solvent molecule can be substituted rapidly — may finally lead to quite high free energies of activation. On the other hand — as will be seen below — successful carrier action *depends* on a quick uptake and release of the metal ion.



$R_1 = R_2 = R_3 = R_4 = CH_3$ Nonactin

$R_1 = R_2 = R_3 = CH_3$ $R_4 = C_2H_5$ Monactin

$R_1 = R_3 = CH_3$ $R_2 = R_4 = C_2H_5$ Dinactin

$R_1 = CH_3$ $R_2 = R_3 = R_4 = C_2H_5$ Trinactin

Fig. 5. Chemical structure of macrotetrolides.

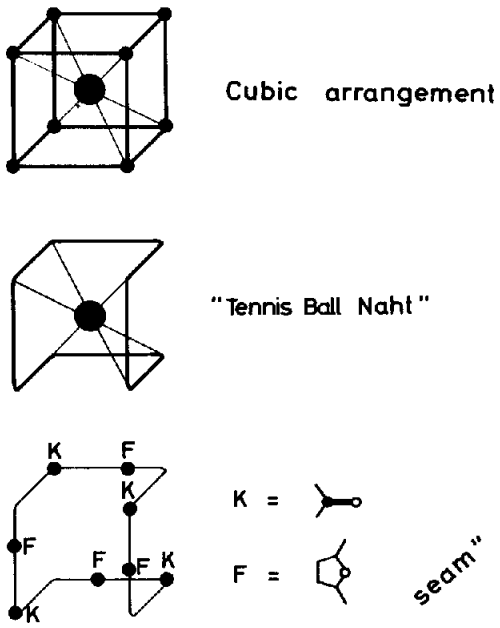


Fig. 6. Simplified spatial structure of metal complexes of the macrotetrolides.

Equilibrium and rate studies have been carried out with monactin and Na^+ as a model system. Monactin belongs to a group of antibiotics which can be isolated from microorganisms such as actinomycetes. The chemical structure as shown in figure 5 was determined by Gerlach and Prelog [5]. X-ray analysis carried out by Dunitz [11] indicates that the metal ion is surrounded by eight oxygens in a quasi-cubic arrangement, whereby the cyclic molecule has a conformation similar to that of a tennis-ball seam (cf. Fig. 6). All polar groups are " inside " the cyclic molecule, whereas the nonpolar groups are situated on the periphery of the complex. In the closed form the macrotetrolide molecule appears to be a hydrophobic sphere. Several problems had to be solved to make such measurements possible.

1. A specific indicator for alkali ions in methanol had to be found. (Since lipophilic carrier molecules are not sufficiently soluble in water, measurements had to be carried out in methanol as solvent.)

2. A relaxation method had to be adjusted to the particular reaction system in methanol. Relaxational amplitudes provide the information about equilibrium parameters such as stability constant, reaction enthalpy etc., whereas relaxation times yield the rate constants for uptake and release of the metal ion.

Ad 1). Murexide [6], the ammonium salt of purpuric acid (Fig. 5) turned out to be an ideal indicator for alkali ions in methanol [7]. A titration curve is shown in figure 6, demonstrating the characteristic colour change upon addition of sodium ions. Figure 7 shows that addition of monactin results in a detectable absorption change. Thus, murexide can be used as an indicator for the reactions of alkali ions with carriers. It is ideal for the present system, because

a) The stability constant of the Na^+-murexide is in a range most suitable for competition with the carrier. (" Half-binding " at concentrations between 10^{-3} and 10^{-4} M.)

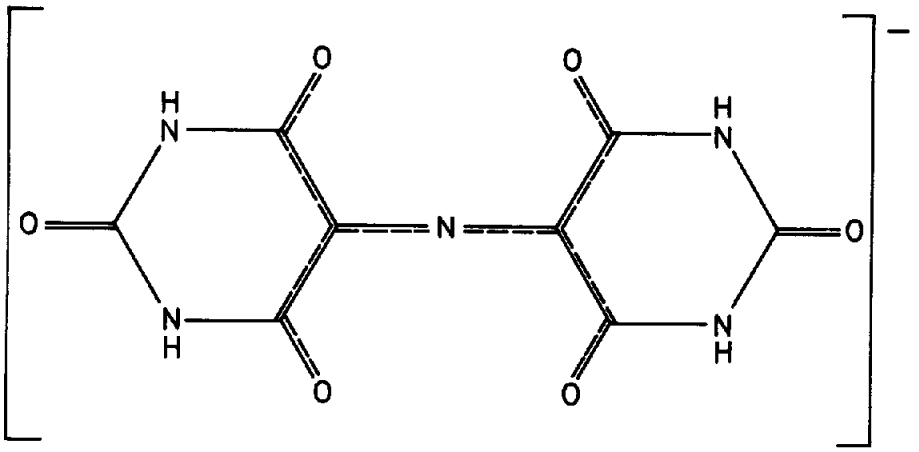


Fig. 7. Chemical structure of the murexide anion.

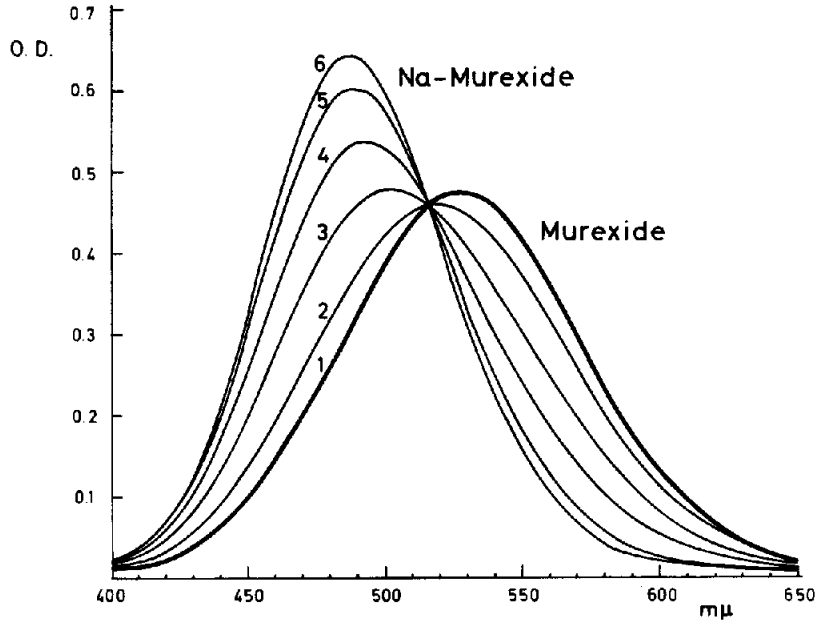


Fig. 8. Spectrophotometric titration of murexide with $Na+$ (25 °C; $c_{Mu} = 4.10^{-5}$ M)

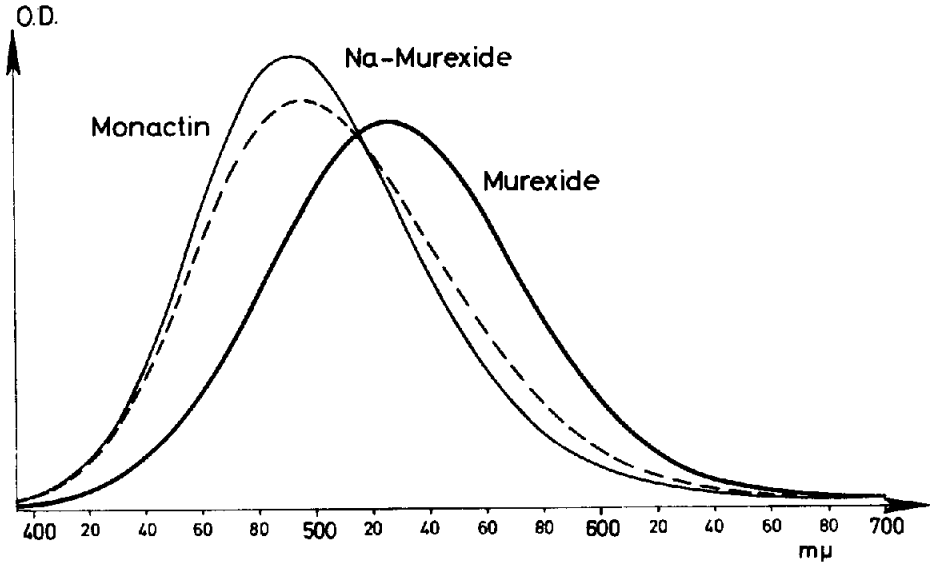


Fig. 9. Decrease of $Na+$-murexide absorption upon addition of monactin (dotted line).

b) The indication is very fast. The reaction of murexide with Na^+ is almost diffusion controlled [7] as found from relaxation studies using an electrical travelling wave technique developed by G. Ilgenfritz [8]. The relaxation time is around 100 nanoseconds.

25° C	$k_R\left[M^{-1}\cdot sec^{-1}\right]$	$k_D\left[sec^{-1}\right]$	$K\left[M^{-1}\right]$
Li^+	$5.5\cdot10^9$	$7.7\cdot10^6$	$7.1\cdot10^2$
Na^+	$1.5\cdot10^{10}$	$5.9\cdot10^6$	$2.55\cdot10^3$
K^+	$\sim2\cdot10^{10}$ *)	$\geq10^7$	$1.1\cdot10^3$

*) diffusioncontrolled

Fig. 10. Stability and rate constants of alkali-murexide-complexes in MeOH.

Figure 8 summarizes the properties of murexide. A detailed description of the measurements is found in ref. [7] (cf. also ref. [4 and 9]).

Dissociation constant of the Na^+ monactin complex :	$K = 2.10^{-3}$ [M]
Reaction enthalpy :	$\Delta H \approx -6.0$ [kcal/mole]
Complex formation rate constant :	$k_R = 3.10^8$ [$M^{-1}\cdot sec^{-1}$]
Life-time of complex :	$1/k_D = 1.5$ [μ sec]

Ad 2). A special technique utilizing differences of temperature jump amplitudes was worked out [7], which allowed a simultaneous determination of stability constants and reaction enthalpies for the metal ion carrier complex. The technique is quite precise and of special advantage in the study of biochemical reactions involving substances which tend to denature. It is described in more detail elsewhere [9].

Using this technique in combination with other relaxation techniques (E-field pulse [8], sound-absorption [10]) for the determination of relaxation times the following results for the system sodium-monactin were obtained.

The most surprising result is the high value for the rate constant of complex formation. A detailed analysis [7] shows that each solvent molecule of the Na^+ solvation shell is substituted within less than 10^{-9} sec. in agreement with the data shown in fig. 1 for single ligand substitution of alkali ions. The mechanism must be a stepwise substitution process, in which each solvent molecule in the coordination shell of the alkali ion is stepwise replaced by the polar groups of the carrier, keeping the total coordination number essentially constant. Such a mechanism requires a quite open form of the carrier molecule in which all the polar groups are easily accessible to the solvated alkali ion. Thus, the uptake of the alkali ion is accompanied by a conformation change of the carrier from an open ring to a closed sphere with the cavity containing the metal ion inside, well shielded by hydrophobic groups. The twisted tennis ball seam structure allows the existence of the two alternate conformations, depending on the compensation of the negative charge of the polar groups by the metal ion.

Four rules for the " design of the carrier " follow from these mechanistic studies :

1. The carrier molecule should possess electrophilic groups which are able to compete with the solvent molecules for metal ion binding. These groups should be located inside an otherwise lipophilic structure, which easily dissolves in membranes.

2. As many solvent molecules of the inner coordination sphere as possible should be replaced by the coordinating sites of the carrier molecule. For two ions of different size the reference state may then involve as much as the total difference of free energy of solvation.

3. The ligand should form a cavity adapted to the size of the metal ion. " Optimal fit " is related to an arrangement where the difference of the free energies of ligand binding and solvation is maximal. It often coincides almost with " fittest " geometrical arrangement. Cavity formation involves ligand - ligand repulsion as well as steric fixation of the chelate.

4. The carrier molecule should possess sufficient flexibility in order to allow for a stepwise substitution of the solvent molecules. Otherwise — i.e. if complete or substantial desolvation would be required for the ion to slip into the cavity — the activation barrier would be rather high and the reaction quite low.

Rule 1 fulfills the biological requirement, i.e. to gate the ion through a (lipid) membrane. Rule 2 and 3 take care of a high selectivity whereas rule 4 allows fast loading and unloading of the carrier. There are not too many low molecular weight structures known which would allow a simultaneous observation of all four rules. Almost all of the classical complexing agents are poor in one or the other respect.

The high rate of complex formation is of great significance for the use of such carriers in biological membranes. The carrier can only be selective if the overall transport rate is not limited by the rate of metal ion release. If this were the case the high selectivity reflected by a high binding constant would be compensated by the slower rate of release. (The ratio of rate constants for uptake and release yield the binding constant). For Na^+-monactin the time constant for release lies in the microsecond range, for the more selectively bound K^+ ion it would reach almost the millisecond range. The release times would be appreciably longer if the recombination process would not have a rate constant of $\sim 10^8\,[M^{-1}.sec^{-1}]$. It turns out that the carrier can play its selective role in alkali ion transport across molecular bilayer membranes only due to its high — almost diffusion controlled — recombination rate.

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De la Physique théorique à la Biologie, C.N.R.S., 1971.

DISCUSSIONS

E.P. KENNEDY : I think the beautiful exposition of Pr. Eigen reveals the high degree of specificity in the interaction of cations with molecules like valinomycin. The comments Pr. Eigen made about the possibility that proteins rather than small molecules might be involved in systems function *in vivo,* should be seriously considered. As we discussed on Tuesday the systems that have been investigated, even though information about them is fragmentary, do require proteins. Whether these proteins have also prosthetic groups which are similar to ionophoric antibiotics still is not clear. However, studies of ion transport in bacteria, especially in *E. coli* reveal that the cell membranes are not freely permeable under all conditions to ions, such as magnesium or potassium ions. If one takes cells of *E. coli* and loads them with radioactive magnesium, then the equilibration of the internal and external magnesium pools is a function of the metabolic state of the cell; that is if the cell has sources of metabolic energy available, it rapidly equilibrates the external and internal magnesium pool. But if one gives energy poisons to these cells, then the equilibration process itself is prevented. This appeared from our study on magnesium transport and studies carried out by Silver in St. Louis on potassium transport. This would argue that you do not have low molecular weight, freely diffusible lipid molecules in the membrane, since these would equilibrate internal and external pools, without regard to the energy state of the cell. At the very least there has to be a system that responds to the energy state of the cell. There has to be regulation and, of course, this very strongly suggests that the apparatus which links the transport system to metabolism is enzymatic in nature.

D. HODGKIN : When I earlier introduced the discussion of the properties of nonactin as an ion carrier, it was not because I thought this was the usual kind of carrier of ions in membranes, but because nonactin had geometrical properties which were known and could be simulated by other systems, perhaps by two or more molecules coming together. I should be very much interested to know whether, in your other model system, more than one molecule is involved in the ion complex.

Journée du 3 juillet 1969

Troisième séance

INFORMATION ET SYSTÈMES BIOLOGIQUES

I - INFORMATION AU NIVEAU MOLÉCULAIRE

PRÉSIDENT C. LEVINTHAL

De la Physique théorique à la Biologie, C.N.R.S., 1971.

INTRODUCTION

C. LEVINTHAL

I've been asked to be the chairman this afternoon since Professor Kachaski was unable to be here.

I'm not sure whether the emphasis of this afternoon's session is going to be primarily on problems of recognition in biological systems or whether we are going to continue to discuss some of the problems of evolutionary biology. However, I will assume that we are going to be mainly interested in various mechanisms involved in different recognition phenomena in biology and some speculation on their evolution. Let me make a very short introduction for those people who may still need it, even though I feel that there are probably very few of them at this meeting.

Until now, we have talked about two mechanisms which are rather well understood in molecular biology whereby objects are able to recognize each other. One of these is recognition by virtue of complementary base sequences on polynucleotide strands. In this case, the recognition takes place by virtue of the self-complementarity of the base pairs : A pairs with T (or U) and G pairs with C. In this way, two complementary polynucleotide sequences will specifically attach to each other after they come close to each other presumably by virtue of random thermal motion. The second general mechanism of recognition is that involved in the three-dimensional complementarity of protein structure. Enzymes can specifically bind substrates, and bodies can specifically bind antigens.

As far as we know, specific binding is a consequence of a complementary, three-dimensional arrangement of atomic groups which allow a large number of weak interaction to sum together to produce a strong force.

Proteins have well-defined three-dimensional shapes, and it is the shape which gives a protein its specific attachment site. In terms of information transfer, it is now clear that the information is transferred from the polynucleotide sequence of the gene to the amino acid sequence of the protein and that the three-dimensional confirmation of the protein is a direct consequence of its amino acid sequence. Thus we can think of the process of evolution as one in which the nucleotide sequence of the gene is selected so that an amino acid sequence of a protein will fold into a shape which has a specific attachment site for some small molecule.

These are the only kinds of specific recognition that have so far been considered in any extensive way by molecular biologists. Whether they will suffice to explain all phenomena of biology is clearly not known but even more importantly, it is not known whether any other kinds of interactions which might arise from specific cooperative forces will in fact be found to play a role in recognition processes of biological phenomena. In this afternoon's session, we will be considering some of the specific phenomena in an attempt to see what types of mechanisms would be needed to understand them.

De la Physique théorique à la Biologie, C.N.R.S., 1971.

INSECT COMMUNICATION BY MEANS OF PHEROMONE MOLECULES

(The example of the silkmoth *Bombyx*)

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Pheromones of the " releaser " type are intraspecific messenger compounds (Karlson and Lüscher, 1959; Wilson and Bossert, 1963, Schneider, 1965, 1969). One individual — the sender — is producing and evaporating the pheromone; another individual — the receiver — senses the pheromone with chemoreceptors and eventually shows a behavioral response. The use of chemical messages in communication between organelles, cells, tissues, organs, and individuals, presumably is one of the oldest means of communication in life.

Accepting our still limited insight into this rapidly expanding field of pheromone communication, it is impossible to survey it in a brief report. I shall therefore concentrate on one specific, but comparatively well known pheromone, the sexual attractant substance of the silkmoth *Bombyx mori*. In many insects — and notably in the moths — the females produce a sexual pheromone in specific glands. This substance is carried by air — either by diffusion or by the wind — to chemoreceptors of the male, which then starts its search for the mate.

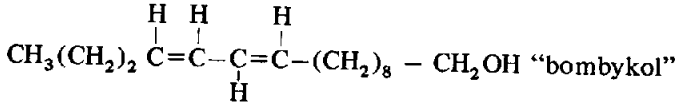
The functional system which has to be analyzed consists of : (i) the productive organ with its morphology, physiology and biochemistry, (ii) the emanation and transfer of the pheromone, (iii) the receptor organ with its morphology, physiology, biochemistry, and biophysics, and (iv) the central nervous system evaluating the receptor organs's messages, and eventually commanding the male's behaviour.

In our example of the silkmoth *Bombyx*, I have to begin my report with the female gland which produces the pheromone and with the chemical composition of this substance which was called bombykol after its identification. Since *Bombyx* is a domesticated animal, transfer is (contrary to the wild moths) no problem in this case with males and females living together. However, molecule transfer over larger distances in the field and animal orientation to odor sources, are not too well understood in general anyway (see Wilson and Bossert, 1963). I will then describe the receptor organ, its structure and function which have been the object of intensive studies in our laboratory. The final

efferent reactions are wing-, and antennal vibrations in *Bombyx,* or the upwind flight of the non domesticated wild moth. Because the neurophysiology of the efferent system is unknown, we will not consider it any further in this context.

The chemistry of the *Bombyx* lure pheromone

The chemical nature of the sexual attractant pheromone of the female *Bombyx mori* was identified as hexadecadien (10-trans, - 12-cis)-ol (1) (Butenandt, Beckmann, Stamm and Hecker, 1959).



The bioassay necessary for this work consisted of behavioral tests with male moths : suprathreshold stimulation of the males leads to a typical vibration of the antennae and wings. The threshold as determined in these experiments favored the assumption that a very small number of molecules is sufficient to stimulate the male insect. Subsequent synthesis of bombykol and its three geometrical isomers (10-cis - 12-trans; 10-cis - 12-cis; 10-trans - 12-trans) permitted tests which revealed that bombykol is maximally effective while the isomers are less potent stimulants, declining in their effectivity according to the listed sequence (Butenandt und Hecker, 1961; Butenandt, Hecker, Hopp and Koch, 1962; Truscheit and Eiter, 1962).

Recently, bombykol was resynthesized and labeled with tritium (Kasang, 1968). This ^{3}H-bombykol enabled us to quantify the pheromone stimulus precisely in behavioral-, and electrophysiological experiments (Schneider, Kasang and Kaissling 1968, Kaissling and Priesner 1970).

The bombykol producing gland

The tissue of the female silkmoth which produces the pheromone, is a specialized area of an intersegmental epidermis near the abdominal tip (Steinbrecht, 1964 b). Lateral areas of this epidermis (the sacculi laterales) are infolded in the resting state but can be expanded by blood pressure thus facilitating the pheromone evaporation (Fig. 1). Histologically, the cells of this tissue are different from normal epidermis cells because they exhibit cytoplasmic inclusions, and a deeply infolded distal cell membrane (Fig. 2), but other typical signs of glandular structure and activity, e.g. extrusions of material, have not been found. As with all arthropod epidermis, the cells are covered by a cuticle which is the secretory product of the cells. The cuticle shows non excretory ducts and is not different from other non sclerotized intersegmental zones. The inclusions in the cells contain a substance of lipid nature, which might be a

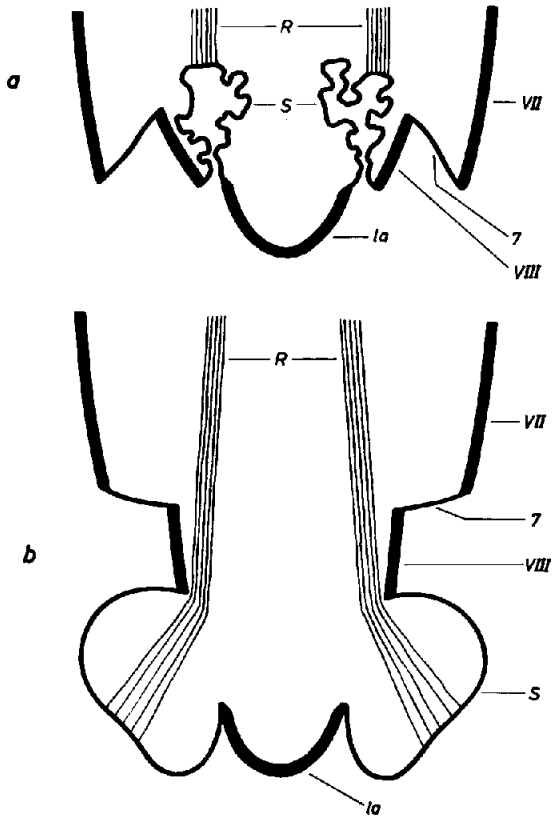


Fig. 1. *Bombyx mori* ♀. Schematic horizontal section through the tip of the abdomen. (a) sacculi laterales (= bombykol producing glands) retracted, and (b) expanded. VII and VIII = 7th and 8th abdominal segment; 7 = intersegmental fold; DE = glandular epithelium; 1A = last abdominal segment; Re = retractor muscles (from Steinbrecht, 1964*b*).

precursor of the final pheromone alcohol which is eventually evaporated at the outermost cuticle layer. We know nothing of the biosynthesis of the bombykol. One reasonable assumption is that the inclusions — which show a UV-absorption similar to bombykol — contain the fatty precursor of the alcohol. This precursor is consequently thought to diffuse through the cytoplasm, the distal cell membrane, and the cuticle. Somewhere on its way, it is possibly enzymatically transformed into bombykol, the sexual attractant.

Histological observations revealed that the number and volume of the lipid droplets grows during the last three days of the moth's pupal life. The droplets are maximally developed soon after emergence and are not much reduced in number and volume two weeks later at the end of the female's adult

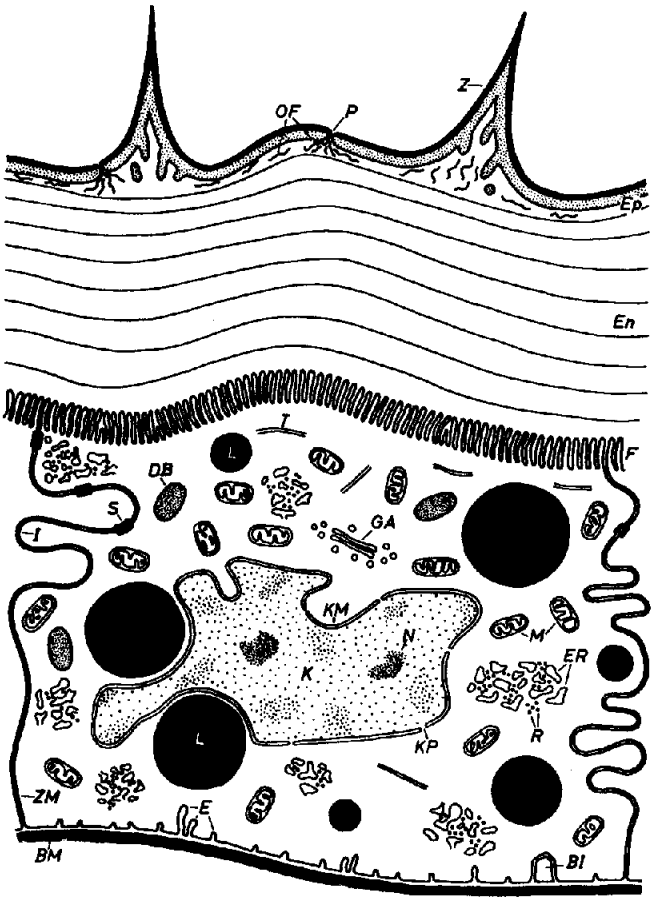


Fig. 2. *Bombyx mori* ♀. Schematic diagram of a longitudinal ultrathin section through a lure gland cell as seen in the electron microscope. BI = Basal interdigitation; BM = basement membrane; D = desmosome; DB = dense body; E = infolding of the basal cell membrane; En = endocuticle; Ep = epicuticle; ER = endoplasmic reticulum; F = fluted distal cell membrane; GA = Golgi apparatus; I = indentation of paired membranes of neigboring cells; K = nucleus; KM = nuclear membrane; KP = pore of the nuclear membrane; L = lipid droplet which probably contains the precursor of the lure pheromone (Bombykol); M = mitochondrion; N = nucleolus; OF = osmiophilic filaments of the epicuticle; P = pore in the epicuticle; ZM = cell membrane. The height of the fluted cell border is approximately 1 µ (From Steinbrecht, 1964*b*).

life. However, the physiological efficiency decreases drastically during the adult life of the insect, thus strengthening the argument that the fatty cell-inclusions are bombykol precursors (Fig. 3).

Finally it was found that the lure effect of a young bombykol gland is similar to that of a 1 cm² piece of filter paper which is loaded with between

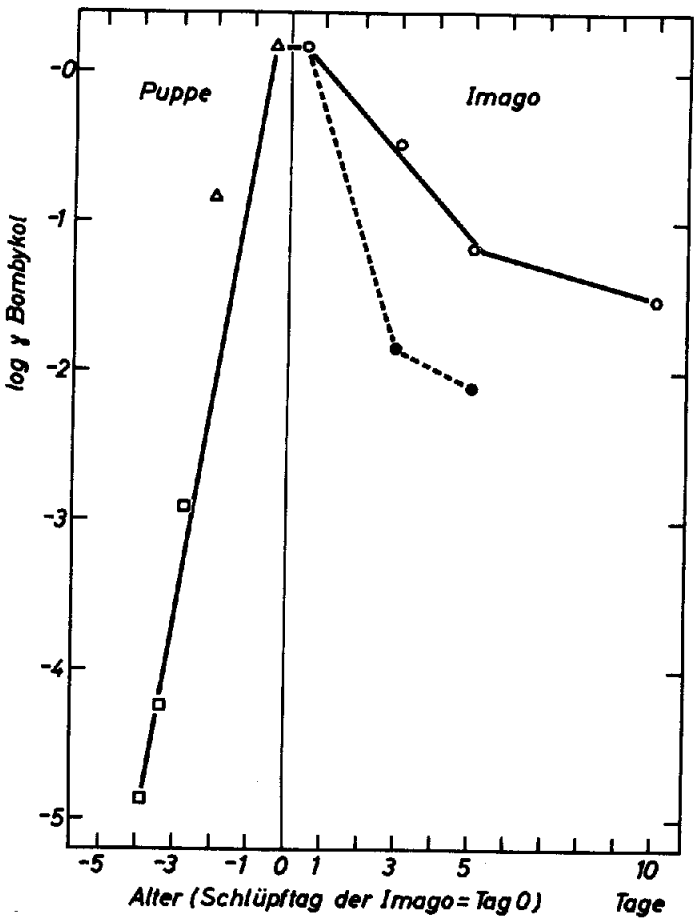


Fig. 3. *Bombyx mori* ♀. Bombykol content of the glands as a function of the developmental state of the animal. Values for the pupal stage (Puppe) are of the " outer activity " as tested by blowing air over the expanded gland to the male moth. Imago (= emerged moth) is the " total (extracted) activity " of the gland. Squares = behavioral tests; Triangles and circles = electrophysiological (EAG) tests. Calibration of the test values was done with the synthetic bombykol. Hatched curve with the imago indicates values with females which were copulated on the day of emergence from the pupa. Ordinate: log µg Bombykol; Abscissa: age of the animals in days (emergence day = day O) (From Steinbrecht, 1964*a*).

1 and 10 µg of the chemically pure pheromone. Also the total quantity of bombykol per lure gland has been determined by measuring the lure effect of filterpaper loaded with gland extracts. Surprisingly the values are in the same order of magnitude as those which were calculated for the bombykol in the sacculus surface (Steinbrecht, 1964 a).

Morphology of the receptors

Many moths, including *Bombyx mori,* have complex combed antennae or feelers. Earlier studies of the animal led to the assumption that these extremities of the insects are the main loci of the olfactory function, including the perception of the sexual attracting scent. Electrophysiological studies with whole antennae and single sensory hairs proved this to be correct.

The *Bombyx* antenna consists of a stem with branches on which are found a multitude of sense organs or sensilla which serve different receptor functions (Fig. 4). The most numerous and impressive structures are cuticular hairs which are between 45 and 140 μ long (Schneider and Kaissling, 1956/57). These hairs (the sensilla trichodea, see fig. 5) are innervated by 1-2 (3) primary sensory cells which were recently found to respond to bombykol (Kaissling and Priesner, 1970). Primary receptor cells of this type have an epidermal cell body or soma, a relatively short and unbranched dendritic process which invades the hair lumen, and a long neurite or axon connecting the soma with nerve cells in the brain. Each neurite reaches the brain via the antennal nerve without branching, fusion, or synapsing (Steinbrecht, 1969*a*). Out of a total of approximately 50 000 fine afferent nerve fibres which leave the antenna, 32 000 or more are from the 16 000 bombykol receptor hairs, at least 15 000 from other odor receptors not sensitive to the pheromone, and the rest from receptors serving other functions.

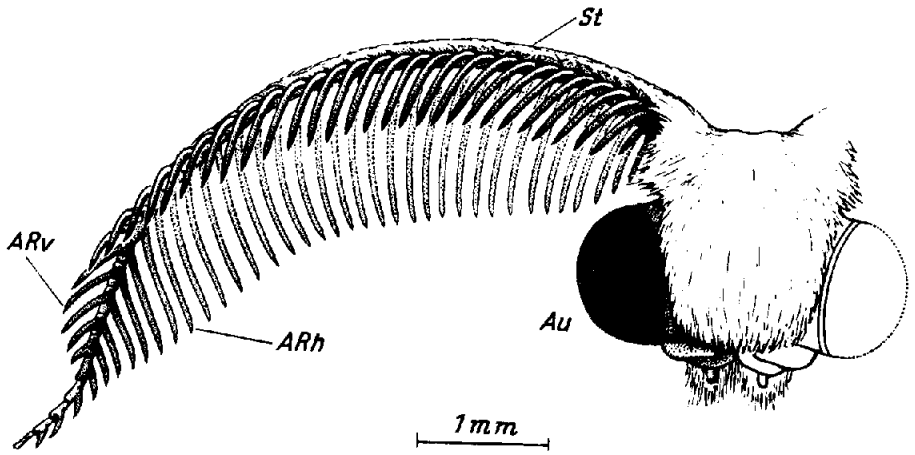


Fig. 4. Frontal view of the silkworm-moth *(Bombyx mori)* head with right antenna (♂ or ♀). Antennal position of the resting animal. The antennal stem with the branches forms a " basket ", of which the opening is turned forward if the male moth is stimulated with bombykol. ARh = posterior row of antennal branches; Arv = anterior row of antennal branches; Au = eye; St = antennal stem. (From Schneider and Kaissling, 1956).

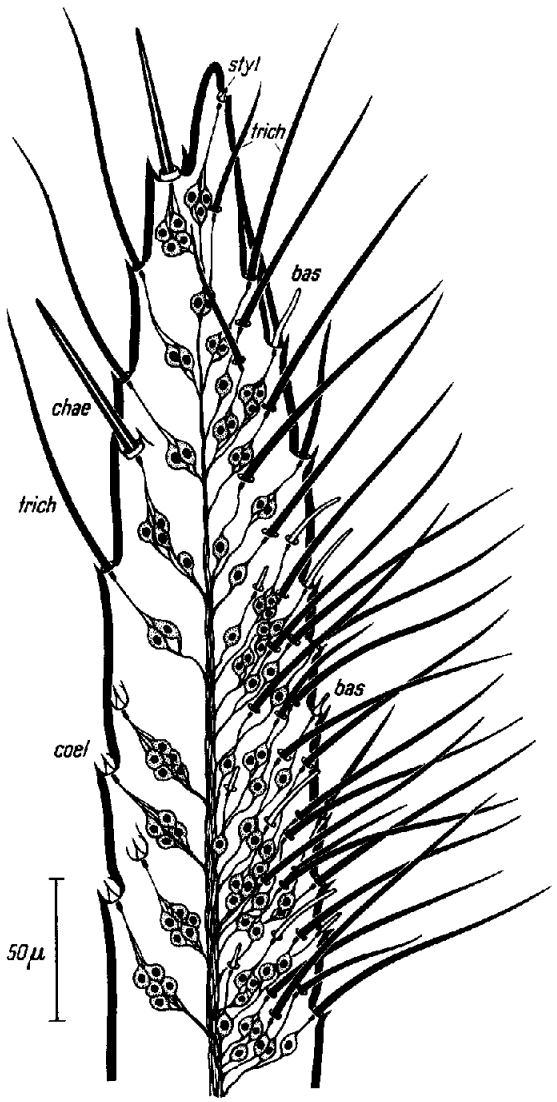


Fig. 5. *Bombyx mori* (♂ or ♀). Schematic drawing of sensilla and nerves at the tip region of an antennal branch. bas = sensilla basiconica ,receptors for general odors); chae = sensilla chaetica (with one mechanoreceptor cell and 3 or 4 cells of unknown contact - chemoreceptor sensitivity); coel = sensilla coeloconica (function not well established, possibly humidity and/or temperature receptors); styl = sensillum styloconicum (function unknown); trich = sensilla trichodea (receptor cells for bombykol in the male moth and for other odorants in the female). The functional determinations are mainly from unpublished experiments by E. Priesner (see also Kaissling and Priesner, 1970), the figure is from Schneider and Kaissling (1957).

It was stated before that the pheromone or its precursor penetrates the sender-organ cuticle (the female gland cuticle) which has no microscopically visible pores. As with the sensory hair cuticle, we are facing the corresponding

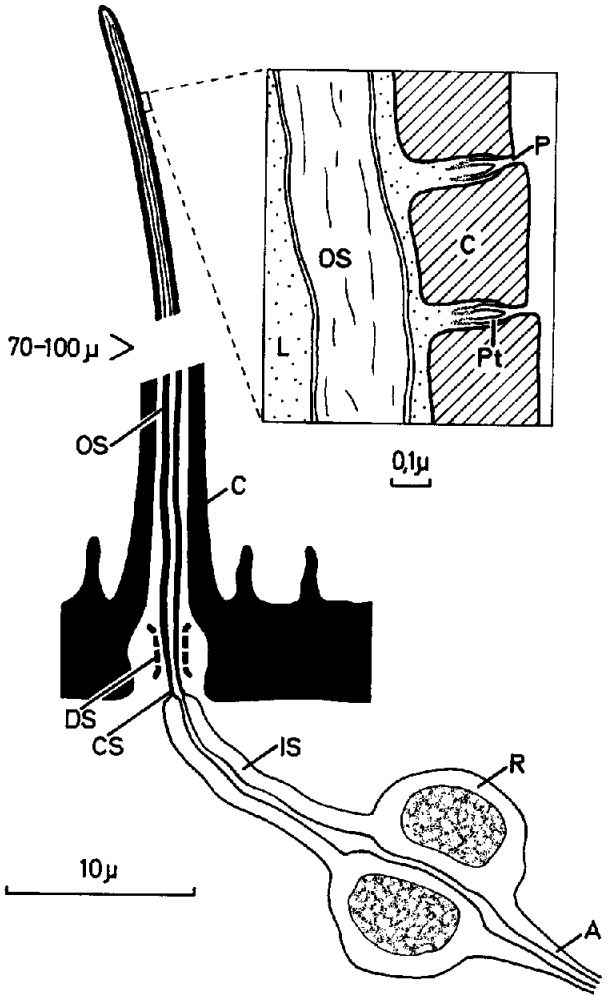


Fig. 6. *Bombyx mori*. Semischematic diagram of a sensillum trichodeum in longitudinal section. Only the basis and the tip of the thick-walled sense hair are shown (approximately 25 % of total hair length). The hair is innervated by two sensory nerve cells which respond to the sexual attractant bombykol. Accessory cells and epidermis cells are omitted. The hair lumen is filled with a fluid, the " sensillum liquor ", which bathes the dendrites.

Inset: enlarger area of the hair wall as seen with the electron microscope.

A = axon; C = cuticle; CS = ciliary zone of the outer dendrite; DS = extracellular sheath of the dendrites; IS = inner segment of the dendrite; L = sensillum liquor; DS = outer segment of the dendrite; P = pore; Pt = pore tubule; R = soma of the receptor cell (Courtesy of Dr. R.A. Steinbrecht).

question on the receiving side of the communication system. Assuming that the stimulating pheromone molecules have to reach the dendritic membrane inside the hair to elicit the response, one is again looking for pores. Recently, these pores have been found and analyzed in detail (Schneider and Steinbrecht, 1968; Steinbrecht, 1969*b*). The hairs of 45-140 μ in length are penetrated by 1000-5000 pores of 150 Å diameter. The pores widen inside the cuticle to a funnel which opens to the hair lumen (Fig. 6). From each pore, a set of several separate pore tubules is seen to reach into the air lumen. In another olfactory sensory peg which was recently studied thoroughly, it was found that the pore tubules are : (i) morphogenetically belonging to the cuticle and not to the dendrite, and (ii) open to the outside (Ernst, 1969). It is reasonable to assume that the odor molecules reach the hair lumen and thus the receptor cell membrane through the pores and pore tubules.

The hair lumen is filled by a liquid, called the " sensillum liquor " (Ernst, 1969). Odorous particles may, after migrating through the tubules, have to diffuse through this fluid before reaching the postulated acceptor loci on the receptor. The total area of all the pores of a bombykol receptor hair of the longer type, is only 1/1000 of the hair surface area which measures approximately 600 μ^2 (Steinbrecht, 1969). This set of pores serves either one, two, or three dendrites, respectively, according to the number of sensory cells innervating the hair.

The arrangement of the hairs on the antenna is such as to filter the odor molecules from the air most effectively. Measurements with ^{3}H-bombykol (Kaissling and Priesner, 1970) revealed that a minimum of 30 % of the odor molecules blown through the antenna is adsorbed on the antennal surface. Theoretical considerations suggest a much higher filter effect because the hairs are spaced sufficiently close to ensure that most of the bombykol molecules hit the cuticule during their thermic movement, when they pass through the antenna (Adam and Delbrück, 1968). Bombykol molecules which hit the antennal surface are adsorbed and do not rebound. This has been shown with the radioactive pheromone (Schneider, Kasang and Kaissling, 1968; Kaissling, 1969*b*).

Receptor function

Animal sensory nerve cells have a receptive membrane area — which presumably is the dendrite membrane in the case of the bombykol receptor cell — and the conducting membrane of the neurite. Receptive membranes are depolarized by adequate stimuli. The depolarization is a function of the stimulus strength and is usually called the receptor potential. Such potentials spread rapidly over the dendritic membrane thus reaching a zone of the neuron's membrane (presumably near the nucleated cell soma) where the nerve impulses

are elicited. Because of their function to generate impulses, receptor potentials of primary sensory cells are also called generator potentials. The stronger the stimulus and the deeper the depolarization, the more impulses are elicited.

More than ten years ago, *Bombyx* antennal physiology has been started by recording the summated receptor potentials of many simultaneously stimulated bombykol receptor hairs. This potential was called the electroantennogram or EAG (Schneider, 1957, 1965, 1969). With the synthetic bombykol and its isomers available subsequently, a stimulus response function was determined. The range from threshold to a presumed sensory saturation reached over several log 10 concentrations of the bombykol source. In this situation, the cis-trans, cis-cis, and trans-trans bombykol-isomers were between 100 and 1000 times less effective than bombykol (Fig. 7) (Boeckh, Kaissling and Schneider, 1965; Schneider, Block, Boeckh and Priesner, 1967).

An absolute threshold could not be determined precisely with these earlier experiments, but some estimates have been made, which now appear to have been close to our latest measurements with ^{3}H-bombykol.

One important object of our experiments with the ^{3}H-bombykol was to quantify our stimulus in absolute units. The question was, (i) how many pheromone molecules are found per volume of air at the locus of the antenna

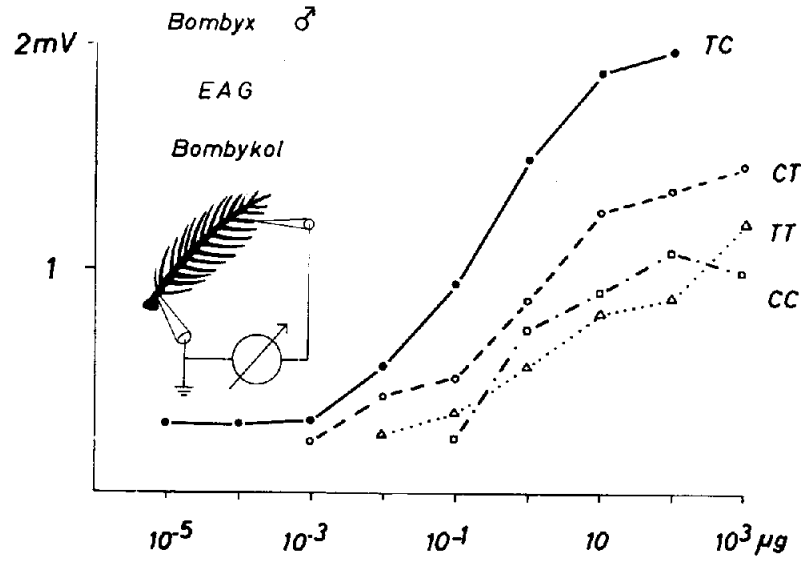


Fig. 7. Electroantennogram amplitudes at different concentrations of bombykol and its isomers. Mean amplitudes of between 30 and 200 individual recordings. TC = hexadeca (10-trans, 12-cis) dien-(1)-ol. CT, CC, and TT are the corresponding geometrical cis-trans, cis-cis, and trans-trans isomers, respectively. The 10^{-5} and 10^{-4} µg bombykol reactions are not different from control reactions. Inset: EAG-recording scheme (modified from Boeckh *et al.*, 1965).

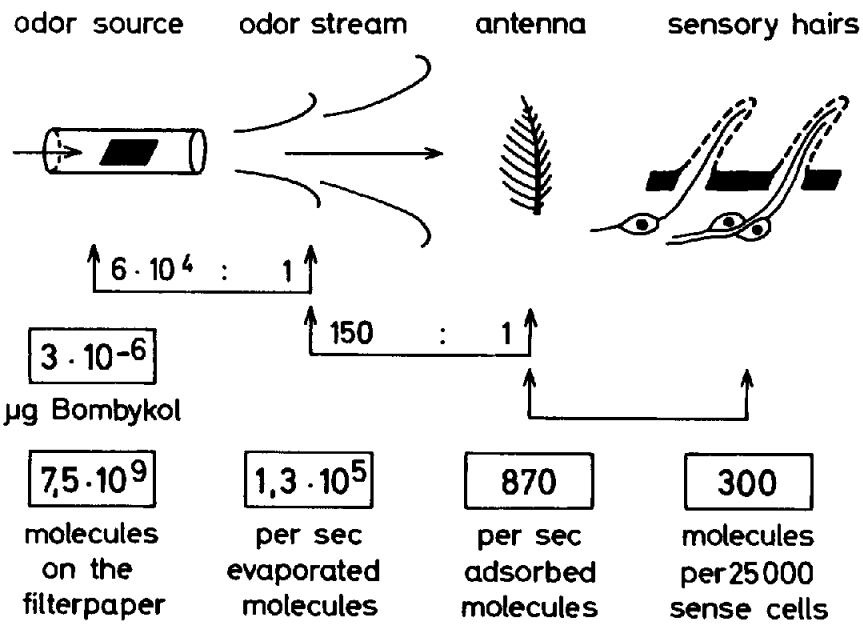


Fig. 8. Radiometric determination of molecule numbers on the antenna of *Bombyx*. Only the number of molecules on the filter paper ($3 \cdot 10^{-6}$ µg bombykol) can be measured directly at the behavior threshold. All other values are extrapolations from measurements at higher intensities. See text for further explanations (Courtesy of Dr. K.E. Kaissling).

when a given physiological response is observed, (ii) how many of the molecules are adsorbed on the antennal surface and on the hairs per unit time.

Fig. 8 explains these measurements and gives the corresponding figures for the behavioral threshold. It is necessary to note in this context that even most sensitive scintillation counters are still too noisy to permit a ^{3}H-bombykol count at the behavior threshold. The data as given in Fig. 8 are therefore the result of an extrapolation over more than three orders of magnitude.

In Fig. 8 it is shown that 25 000 receptor cells (of the more sensitive type) on one antenna receive 300 bombykol molecules with a threshold stimulus. This number is calculated with the assumption that all the molecules which hit a sensory hair are effective. (If we relate this number to only the area of the pores of a hair, the figure has to be divided by the factor 1000 !)

The consequence of the assumption that the whole hair surface is the molecule catching area is that we have to count on surface diffusion to play an important rôle. Again, theoretical aspects are in favor of such a process which would also be fast enough to fit to the response time (Adam and Delbrück, 1968). The sequence of events is now : bombykol molecules are carried by air to the antenna, strike the hair cuticle where they adsorb, reach the pores by surface diffusion, continue to migrate through the pores and pore

tubules to the sensillum liquor, from where they eventually reach the dendritic membrane. This picture of causal events is — with the exception of the adsorption — only indicative or hypothetical but nonetheless highly plausible. We are sure of the first and the final step which are the hair's molecule-catching activity and the electrical membrane response. The assumption of the diffusion processes if hypothetical but reasonable, and also physically possible in less than the reaction time (Adam and Delbrück, 1968; Kaissling and Priesner, 1970).

While these threshold determinations demonstrate the extreme sensitivity, it needs to be clarified whether one bombykol molecule hit is the minimum stimulus for a cell to signal to the brain. Could it be that a statistically sufficient number of cells is hit by two molecules in " physiological synchrony " ? This question could recently be answered by Poisson statistics applied to a new series of bombykol-behavior experiments (Kaissling and Priesner, 1970). If air is blown over a filter paper which contains $3 \cdot 10^{-6}$ µg of bombykol, 22 % of the male moths react in the typical manner. The calculation of molecule adsorption as given in Fig. 8 permits us to state that under these circumstances, 1.25 % or 300 of 25 000 receptor cells of one antenna receive one or more hits, and approximately 0.005 % of the cells receive 2 or more hits. Because

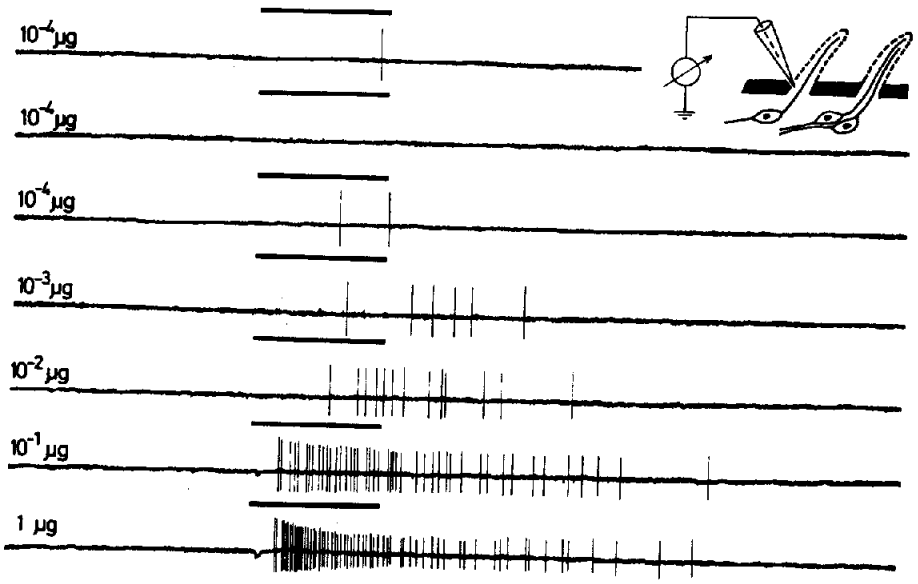


Fig. 9. *Bombyx mori* ♂. Impulse reactions of a tonically reacting bombykol receptor cell. Capillary microelectrode placed at the base of a sensillum trichodeum. With a bombykol load of 10^{-4} µg on the odor source (see the cartridge in fig. 8) the cell may or may not react. With higher concentrations, the cell always reacts, the more frequently, the stronger the stimulus. Black bars above record give stimulus time (1 second). Impulse amplitude approximately 1 millivolt. Extracellular recording.
Inset: recording scheme (Courtesy of Dr. E. Priesner).

the value of 22 % reacting animals is significantly different from control ($p = 0.002$), it can be stated that it suffices for the bombykol receptor cell to receive one molecule (" quantum ") hit to signal this event to the brain. Such a calculation shows in addition, that with a stimulus concentration where we have in the average one molecule hit per second and each of the sense cells, already 100 % of the animals are reacting.

While the claim seems to be well established that single bombykol molecules are not only able to elicit a possible local effect, but must be able to elicit membrane effects which are sufficient to signal this event to the brain, one would like to know how many impulses are elicited by one molecule. Does the electrophysiological recording show us signs of activity with a pheromone concentration near threshold? The EAG-threshold as described above, is certainly much too high. Single cell responses, however, should permit us to test the one hit argument. Such recordings have recently been successful and are shown in Fig. 9 in original recordings (Kaissling and Priesner, 1970). One can now plot the number of cells responding with one or more impulses as a function of the stimulus intensity, and finds that the resulting curve is clearly

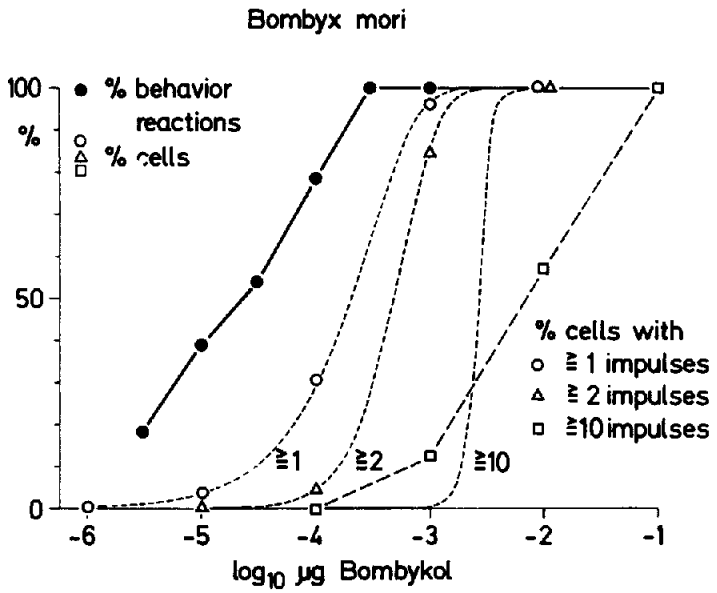


Fig. 10. *Bombyx mori* ♂. Behavior reactions and nerve impulses of single receptor cells. — Stimulus: bombykol in µg on the odor source (filter paper, see fig. 8). Behavior reactions counted during two seconds. 22 % of the animals react at $3 \cdot 10^{-6}$ µg of bombykol ($p = 0.002$). — The percentage of cells firing ≧ 1 and ≧ 2 impulses (hatched curves) fits the theoretical expectation of the corresponding poisson distribution. The 10-impulses curves are not fitting. Each impulse value is the mean of approximately 800 measurements. See text for additional explanation (Courtesy of Drs. K.E. Kaissling and E. Priesner).

identical with a one hit Poisson curve (Fig. 10). The corresponding curve for the cells as responding with two or more impulses is identical with the two hit Poisson curve, but the curve for cells responding with 10 or more impulses, is no more in agreement with a Poisson distribution.

Because of the good agreement of the experimental impulse data and the random (Poisson) distribution, it is now possible to deduce the impulse number at the behavior threshold by extrapolation: 200 bombykol induced impulses are fired by receptors of the sensitive type at a stimulus intensity of $3 \cdot 10^{-6}$ µg of bombykol. This number is in the same range as the number of 300 bombykol molecules which are adsorbed on the receptor hairs during the same threshold stimulus. Obviously, nearly all of the molecules which are caught by the hair surface, are effective stimulants for the receptor cell, and one single molecule suffices to elicit one single impulse. The cells consequently are molecule counting devices at low stimulus intensities and the minimum stimulus claim that " one molecule of bombykol elicits a significant cell signal ", is verified.

With this statement we can now return to the minimum behavioral reaction. It as said that a minimum of 200 cells has to fire one nerve impulse each to elicit a significant behavioral response. This now brings us to another point. Sensory systems — as is well known from research on vision — are necessarily noisy. What actually counts, is the signal to noise ratio. As for the periphery of the bombykol sensing system, it has been found that the whole set of 25 000 receptors of one antenna is signaling in the resting state with a maximum of 1600 nerve impulses per second (Kaissling and Priesner, 1970). This is the noise, the insect's brain is dealing with. An increase of this firing rate by 200 impulses/sec (the suprathreshold bombykol response) seems to be a reasonable value to be detectable by the central organ.

There are two types of bombykol receptor cells as combined with the sensilla trichodea. One is a tonically responding and very sensitive one (80 % of the receptors), as described so far. The other is responding phasically, is much less numerous (20 % of the receptors) and responds in a range of stimulus concentrations, where we recorded the EAG before (Priesner, 1969).

Both receptors respond to bombykol, its isomers, and a number of other substances (see also Schneider, 1965). However, only bombykol is effective in minimum concentrations. To simulate a certain electrophysiological bombykol response, one needs up to millions of times more molecules if for instance terpineol is the stimulus. This discrepancy is not understood, as is the specificity and the principle of transduction in olfactory receptors.

If one assumes speculatively that the dendritic membrane of the bombykol receptor cell consists of a mosaic of structure protein molecules, which are the acceptors for the pheromone particles, one hit of the stimulating particle must suffice to elicit a depolarization which is sufficient to generate one nerve impulse. This means that the system is a highly triggered one.

We are at present studying this function. Rise-, and decaytime constants of the summated receptor potentials, the EAG, could be treated with a mathematical formalism which is in agreement with either an adsorptive or a simple enzymatic process (Kaissling, 1969*a*). With this formalism one can tentatively calculate the number of bombykol acceptors participating in the transducer process.

In addition, we are trying to analyze the fate of the bombykol molecule after it's contact with the receptor. One attractive possibility is that bombykol is not changed during the transduction process proper, but we have no clear cut evidence in favor of this yet. We have, however, evidence for the assumption that bombykol which is adsorbed elsewhere on the male moth's body — including the antenna — is slowly metabolized, which is not the case with bombykol as adsorbed for instance on cotton fibres (Kasang, 1969). Such a mechanism would be meaningful because it ensures that only the newly arriving molecules which carry actual information from the sender to the receiver, are eliciting a response. The molecules which are not rapidly reaching the receptor membrane, would sooner or later be chemically changed and have no chance of giving misleading information.

Incidentally, also the body surface of the female moth is able to metabolize the bombykol. The meaning of this could be that the active evaporation of bombykol from the expanded pheromone gland is the only effective pheromone source in normal life. Any bombykol contamination of other parts of the female's body would be eliminated as a possible secondary bombykol source by these metabolic processes. With wild moths it was observed that the females became rapidly non attractive for the males if they were retracting their glands and thus stopped " calling " their mates (Brady and Smithwick, 1968; Götz, 1951; Jacobson, 1965; Shorey and Gaston, 1967).

Résumé

The best known pheromone communication system is the sexual attraction in the silkmoth *Bombyx mori*. We have been studying the production and sensory reception of the attractant alcohol bombykol. This substance has recently been resynthesized in tritiated form.

The productive system is an abdominal gland in the female insect. The gland cells contain inclusions which presumably are precursors of the alcohol which eventually evaporates from the gland's cuticle.

The receptor system consists of odor receptor cells on the antenna of the male moth. These receptor cells are primary sensory nerve cells which are extending their receptive processes — the dendrites — into cuticular hairs of between 45 and 140 μ length. The dendrites are bathed in a fluid, the sensillum liquor, which is found in the lumen of the hairs. This hair lumen is connected

with the outside by a system of 150 Å diameter pores and pore tubules which extend into the liquor to the dendrites. The pores and tubules are open to the outside.

The long process of the receptor cell, the neurite or axon, connects the receptor system with the brain.

The receptor system can be analyzed by a study of the behavior reaction: male moths vibrate their wings and antennae if air containing bombykol is blown to them. Electrophysiological tests of whole antennae reveal a slow overall response — the electroantennogram or EAG — which is thought to be the sum of many primary receptor potentials. In addition, extracellular single receptor responses are recorded with microelectrodes from the basis of the hairs.

^{3}H-bombykol permitted us to quantify the stimulus precisely. At the behavioral threshold — with 22 % of the male moths reacting — the 16 000 hairs with a surface of 600 μ^2 and two sense cells each, receive a total of several hundred molecule strikes per stimulus time. With this low value, and our knowledge that bombykol readily adsorbs at the antenna, we can exclude the assumption that only the molecules which hit the pores directly, are effective stimulants. The area of the pore openings is only 1/1000 of the hair surface, an area much too small to be sufficient for the effect. This leads to the conclusion that the whole hair surface is the catching (" receptive ") area and that the molecules migrate by surface diffusion to the pores and via the pore tubules to the liquor and the dendritic membrane.

Poisson statistics applied to the behavior response data prove that one single molecule hit is sufficient for the bombykol receptor cell to form a meaningful signal for the brain. This is in agreement with the single cell recordings. If one plots the percentage of cells responding with one, or two impulses, respectively, as a function of the molecules adsorbed on the hairs with increasing stimulus strength, these curves fall in line with the poisson-curves. The receptors, therefore, are molecule counting devices as long as the stimulus concentration is low. One molecule elicits one, and two elicit two impulses. But with 10 or more molecules hitting the cell per unit time, the response is not proportional to the number of molecules.

Such a system appears to be highly triggered. The mechanism of the information transfer from the bombykol molecule to the postulated acceptor molecule in the receptor membrane, is unknown, as is transduction in other sensory receptors. The rise and decay timecourse of the first electrical receptor reaction allows a formalistic mathematical approach which is in agreement with either an enzymatic or an adsorptive process. We are at present still ignorant of the fate of the bombykol molecule after it transfered its message to the acceptor.

Acknowledgments

The observations, calculations, and speculations which have been presented in this review are the result of the closely correlated teamwork of a group of scientists and technicians. The goal of our work is to analyze insect olfaction in general and bombykol reception in particular. Recently, the latter complex of problems has been worked on successfully by Drs. K.E. Kaissling (Physiology), G. Kasang (Chemistry), E. Priesner (Physiology), and R. A. Steinbrecht (Histology). We all are — last but not least — indebted to our technical staff members for their skillful assistance.

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DISCUSSIONS

J. MONOD : How do you fell that the insect (male) gets oriented ?

D. SCHNEIDER : There is indicative evidence for the following mechanism : With low stimulus intensities, the pheromone molecules are only a " go " signal, which makes the male moth fly upwind. It is another problem, how the animal decides upon the upwind direction. It probably does this optomotorically. Experiments related to this orientation are not easy to do and have never been done on a sufficiently large scale. Near the odor source, the spacing of the two antennae may help the animal to orient in relation to the odor concentration gradient.

S. BENNETT : My question, Professor Schneider, relates to the events which transpire between the moment that your molecule hits a point on the surface of the antenna and the time that it finds its way into the boom of the pit. You postulated that one molecule hitting anywhere on the surface would trigger off a response of the nerve. And you postulated also some sort of a diffusion or migration along the surface.

D. SCHNEIDER : We are forced to count on a diffusion process to bring the adsorbed molecules eventually to the dendrite. Without such a process, our threshold would be too low.

S. BENNETT : Do you envision this as a random diffusion process, or is there some unexpected directional component, perhaps even a pumping, or is there some sort of a current set up in the components of the cuticle which might carry these molecules ? Need one postulate a source and a sink, which would provide a stream which would increase the probability or decrease the time interval between the moment of impact of molecule on cuticle on the one hand and its arrival at the actual membrane excitatory site on the other ?

D. SCHNEIDER : Adam and Delbrück (1968) predicted such a process, which is random and fast enough to fit to the observed reaction times. So far we do not " need " any directional transport mechanism of any kind.

F. LYNEN : Do you know whether the aldehyde of bombykol is active too ?

D. SCHNEIDER : We have not tested the bombykol aldehyde yet. The dihydro-bombykol (with only one double bond) is much less effective than bombykol or the geometrical bombykol isomers, but much more effective than e.g. terpineol.

F. LYNEN : I am just wondering because you are considering an enzymatic reaction to be involved. In that context one should consider the possibility of the oxydation of the alcohol to the aldehyde. To pose a direct question : If you give a mixture of alcohol and aldehyde does the aldehyde inhibit the response to the alcohol.

D. SCHNEIDER : It would be interesting to do this experiment but the bombykol aldehyde has not been available for experimentation yet.

M.U. PALMA : I should like to know if this particular (and fascinating) system of communication is in general use for other kinds of messages, for other substances and for other receptive organs. I know, for instance ,that whenever some ants go across an area over which another ant has been killed, they immediately panic.

D. SCHNEIDER : Ants do not really panic, but they become very excited and run about until they detect the agressor which they attack. — Some schooling fishes have an alarm substance which is released if their skin is mutilated, for instance by a predator. This reaction is some kind of " panic " because the school immediately hides at the bottom. Again, the alarm pheromone is detected by olfactory receptors.

A. FESSARD : In the case of an asymmetric reception by the two antennae, does this give a clue for a directional reaction ? If the odor comes from the right side for instance ?

D. SCHNEIDER : This was found to be true in the honey bee (Lindauer and Martin, 1963). In *Bombyx* I would think that the less sensitive (fastly and phasically reacting) receptor type would be a candidate to steer such a reaction. The very sensitive, slowly and tonically reacting bombykol receptor probably only gives the order for the upwind flight. This receptor is out of function with higher bombykol concentrations. But such experiments have not as yet been done with any moth.

A. FESSARD : Another question. Could you follow the message to the higher central ganglia or cephalic ganglia ?

D. SCHNEIDER : We have not done such experiments with neurophysiological methods. But Dr. E. Kramer in our laboratory works on a cybernetical model experiment with honey bees. The results are partly corroborating earlier concepts (upwind movement under odor stimulus) but too complex in detail to be explained here in brief.

S. EBASHI : Do you have any information about the ionic composition of the fluid and particular ions responsible for the change in the membrane potential ?

D. SCHNEIDER : The amount of " sensillum liquor " bathing the dendrite in the hair lumen is so small that we have no hope to determine its composition. This determination should be done but we don't know how to do it.

S. EBASHI : How about the effect of bombykol on other tissues, for example, other nerve cells, or other muscles ? This would give some information about the nature of the membrane process in the receptor.

D. SCHNEIDER : We have only tested bombykol on the antennal receptors of other moths. The effect declines with decreasing systematic relationship.

De la Physique théorique à la Biologie, C.N.R.S., 1971.

ANTIBODIES : A MOLECULAR RECOGNITION SYSTEM *

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Classical studies of the immune response have shown that introduction of an antigenic molecule into vertebrate organisms elicits the synthesis of antibodies that can bind specifically to that antigen [1]. The variety of chemically different antigenic structures is enormous and the capacity of the immune system to respond to so wide a range of stereochemically distinct molecules is as astonishing as the specificity with which each antigen is bound. Moreover, upon a second encounter with the same antigen, there is immunological memory, i.e. the specific response is more rapid and larger amounts of specific antibodies are synthesized. Thus antibodies constitute a molecular recognition system which is remarkable for its specificity, range of response, and control [2, 3].

Analysis of the thermodynamics and mechanisms of antigen binding by antibody molecules has been the preoccupation of many immunologists in the past [4]. From their studies, it is clear that the binding depends upon the close complementarity of the shapes of the antigen and the antigen binding site on the antibody molecule. This complementarity (Fig. 1) has been deduced from cross reactions with related antigens and the nature of the binding : the free energy ranges from approximately — 4 to — 12 kcal/mole, and hydrophobic interactions, hydrogen bonds, electrostatic interactions and steric repulsion contribute to varying degrees, depending on the particular antigen-antibody reaction. Because this type of binding does not differ from that between enzymes and substrates, it is tempting to think of antibodies in the same way as one thinks of enzymes.

There are, however, several features of the immune response which indicate that this analogy holds only in the most superficial sense. First of all, unlike most substrates for enzymes, many antigens may be constructed which never existed in the evolutionary history of the organism [1]. Moreover, antibodies may be elicited by injecting as antigens practically any protein molecule from a variety of animal species, and it is not likely that all of these protein antigens played a direct role in natural selection during the evolution

* Supported by grant GB 8371 from the National Science Foundation and by grant AM 04256 from the National Institutes of Health.

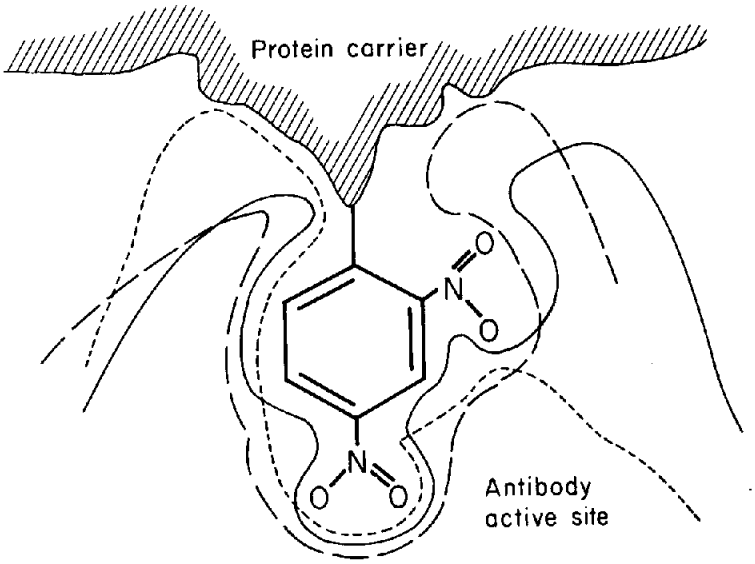

Fig. 1. Diagram illustrating the complementarity between antibody and a small antigen or hapten (the dinitrophenyl group) coupled to a protein carrier. The various contours of the antibody active site indicate the greater or lesser degrees of fit between different antibody molecules and the same antigen.

of the immune response. Furthermore, unlike the induction of enzymes by substrates, a single antigen usually elicits the synthesis of a large population [5] of different antibody molecules which fit the antigen more or less well (see fig. 1). Thus the antibody response is degenerate, i.e. there is a many-one relationship between specific antibodies and any particular antigen and no single " best " antibody has evolved for each of the different three-dimensional antigenic structures.

A typical sequence of events following injection of an antigen [6] is schematized in Figure 2.

Early attempts to explain antibody production resulted in the formulation of a number of so-called instructive theories. The fundamental notion underlying these theories was that information on the three-dimensional structure of the antigen was necessary at some stage of synthesis and folding of the polypeptide chains of the antibody molecule. In their simplest forms [7], instructive theories suggested that the antigen served as a template around which was folded that portion of the antibody polypeptide chain destined to become the antigen binding site.

Instructive theories have now been abandoned, largely because the results of analyses of the structure of antibody molecules and of the cellular dynamics of the immune response have made them untenable. They have been supplanted by selective theories [8, 9] which state that the information required for

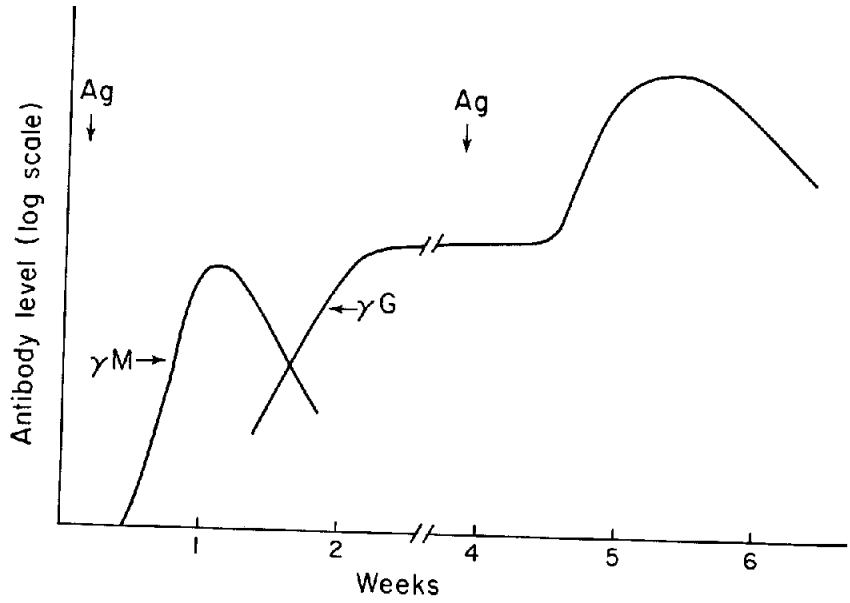

Fig. 2. Sequence of the antibody response to two injections of antigen (Ag) spaced about three weeks apart. The antibody level is measured in the serum of the animal and two classes of immunoglobulin are elicited: γM immunoglobulin and γG immunoglobulin. The second antigenic stimulus provokes an accelerated and increased production of specific γG immunoglobulin (" immunologic memory ").

specificity and range *already* resides in the organism before exposure to the antigen. The antigen serves to stimulate (or select) only those cells that synthesize complementary antibodies. As shown in Figure 3, selective theories require a minimum of three conditions : 1) the organism must contain information for the synthesis of an *enormous* repertoire of different antigen binding sites most of which may never be used, 2) there must be a mechanism for antigen trapping to favor encounter with the appropriate lymphoid cells, 3) lymphoid cells must constitute an amplifier of high gain which is triggered by the antigen, so that after selection of the appropriate cells, a significant number of antibody molecules of the correct specificity are produced. This is realized by cell division and increased antibody synthesis [3].

Such a system has a number of interesting properties. It is clear, for example, that the specificity of the antigen recognition function (ARF) is a property of the entire system rather than of single antibody molecules alone. Moreover the system is clonal, i.e. each cell synthesizes a single variety of antibody and after encounter with the appropriate complementary antigen is stimulated to divide and form progeny cells which synthesize the identical type of antibody at greatly increased rates [3, 9]. The antibodies released can now bind antigen in the fluid spaces of the organism, or bind to cells, and they

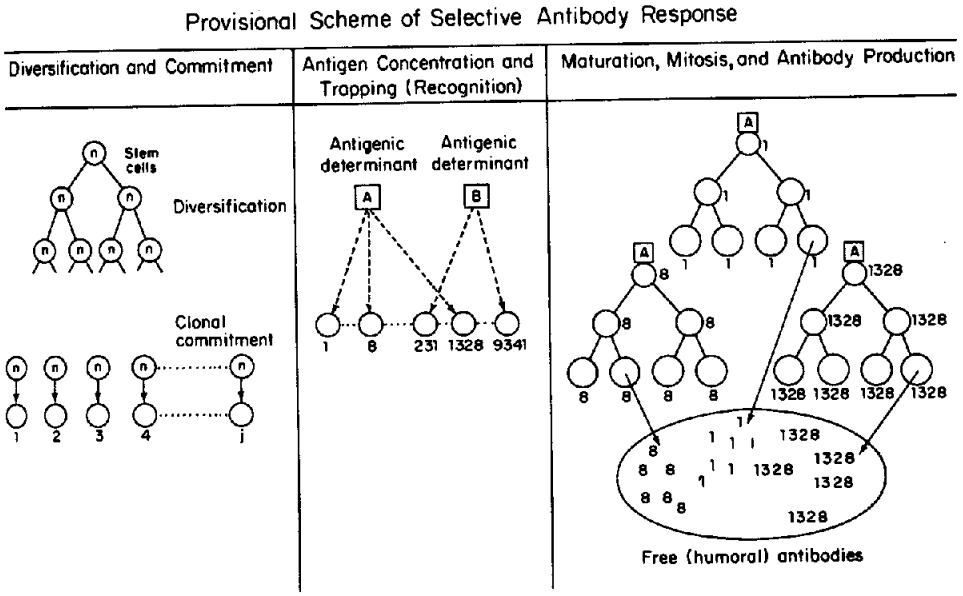

Fig. 3. Scheme of events in a selective antibody response. The lower case " n " inside stem cells indicates that they are uncommitted and pluripotential. For clarity, diversification and commitment are presented as separate events, although a detailed model of differentiation from stem cells is not intended. Arabic numbers outside each cell represent the unique immunoglobulin produced after clonal commitment. These " receptor " antibodies can interact with various antigenic determinants. The degeneracy of the immune response is indicated by recognition of a single antigenic determinant (e.g. A or B) by several different cells which produce different antibodies. Interaction of a committed cell with antigen stimulates maturation, mitosis, and increased humoral antibody production (amplification).

can also carry out a number of effector functions (EF) which are essential in the immune response but which do not depend on the specificity of antigen binding. These include complement fixation, skin fixation, opsonization, and placental transfer. Particular structures on antibody molecules may function in triggering of mitogenesis and cell maturation after interaction with the antigen (see fig. 3). These structures, which have not so far been identified, may also play a part in immune tolerance which is the specific failure of the cells to respond to a given antigen.

Although this is a qualitatively satisfying picture of a selective molecular recognition system, we lack detailed information on the number of different specificities and the mechanism of their generation, on the efficiency of each process, and on the quantitative features of control required for the whole system to function [10]. Some information on these mechanisms has come from studies of the molecular structure of antibodies and the remainder of this paper will deal with this subject.

My remarks will be restricted to one class of antibodies, the so-called γG immunoglobulins, for there are good reasons to believe that this most prevalent class is representative. We now know the covalent structure of an entire γG immunoglobulin molecule [11]. Although its three-dimensional structure and the details of the antigen-binding sites remain to be determined, we can say a good deal about the relation of structure to function by comparing the entire amino acid sequence with sequences of portions of other antibody molecules analyzed by other workers [2, 3]. This is possible because the three-dimensional structure of the molecule and the combining sites depends upon the amino acid sequence of its polypeptide chains [12]. In addition to providing information on the nature of the diversity of antigen combining sites, the structural data from a variety of laboratories [2, 3] suggest clues to the mechanism of clonal response and the mechanisms of differentiation of the immune response. The data on the amino acid sequence also provides evidence on the evolutionary origin of the antibody molecule.

The organization of a human γG immunoglobulin molecule [11] is shown schematically in Figure 4. The structure contains two identical light polypeptide chains and two identical heavy chains. Each light chain is linked to its neighboring heavy chain by noncovalent interactions and by a disulfide bond consisting of half-cystines V of each chain. Half-cystines VI and VII of the heavy chain form the two bonds linking these chains and the half-molecules

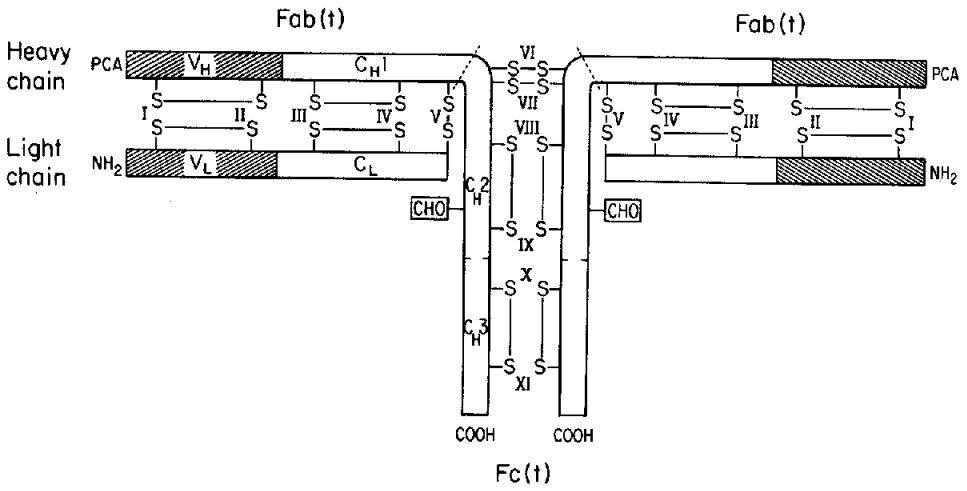

Fig. 4. Structure of a human γG1 immunoglobulin molecule from myeloma patient Eu [11]. Site of cleavage by trypsin to form Fab(t) and Fc(t) fragments is indicated by dotted lines. Half-cystines contributing to disulfide bonds are designated by Roman numerals. V_H and V_L - variable regions of heavy and light chains. C_L - constant region of light chain. C_H1, C_H2, and C_H3 - homology regions in constant region of heavy chain. PCA - pyrollidone carboxylic acid. CHO - carbohydrate.

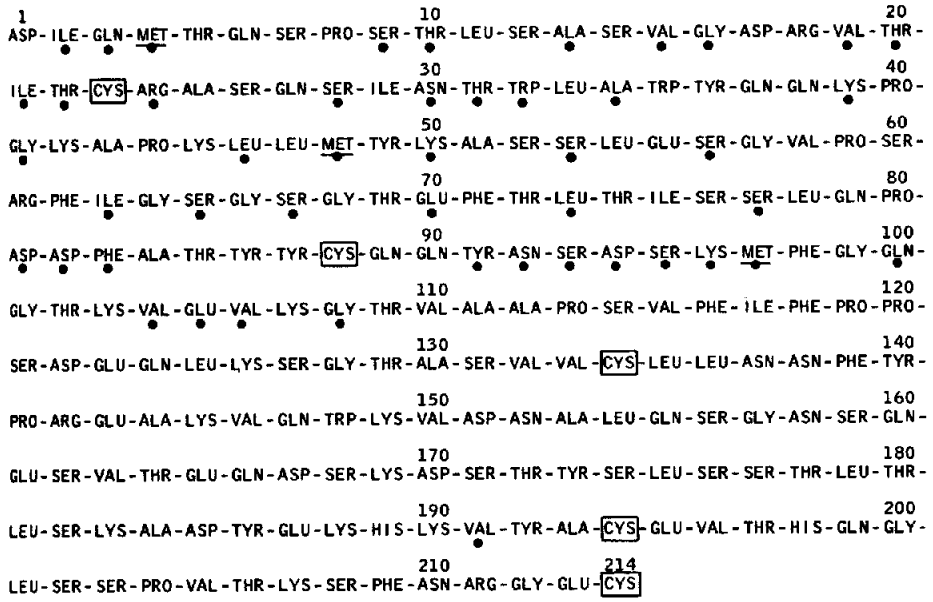

Fig. 5. Amino acid sequence of light chain of Eu. Positions in which amino acids differ in κ chains of the same subgroup [see Refs. 2, 3] are indicated by dots under the appropriate residues. The V_L region extends to residue 108. Variant position at position 191 is known to represent genetic polymorphism of the constant region, C_L.

together. Limited hydrolysis of the molecule with proteolytic enzymes such as trypsin cleaves the molecule into Fab fragments which contain the antigen-combining sites and Fc fragments which mediate certain effector functions. The disulfide bonds are linearly and periodically disposed in both chains and it is striking that the light and heavy chains can be aligned so that their disulfide bonds are in register. This arrangement has implications for the evolution and function of the molecule which will be discussed later.

The amino acid sequence of the light chain is shown in Figure 5. Comparison of the sequence with that of free homogeneous light chains, which are known as Bence-Jones proteins [see 2, 3, and 13], reveals an unusual structure. The first 108 residues have 43 positions in which given amino acids may be replaced by others in different molecules whereas the last 106 residues show a replacement only at position 191. Thus the chain may be divided into a variable or V region and a constant or C region. The replacement in the C region at position 191 is a genetic polymorphism similar to that found in other proteins. The evidence indicates that a single gene specifies the C region and thus, for example, some individuals may be homozygous for valine or leucine at position 191.

The situation is more complicated in the V region. No genetic markers have been identified but examination of various proteins has indicated that V

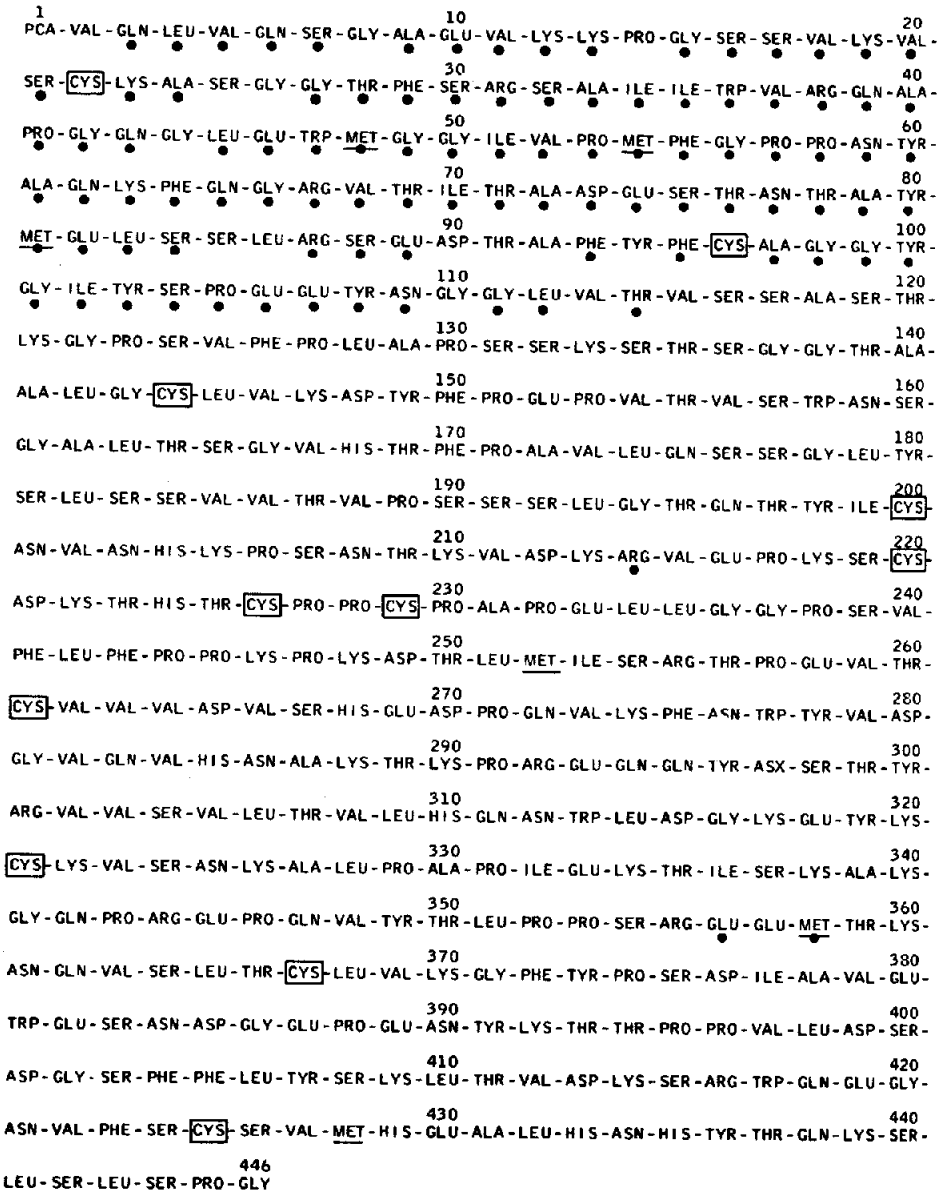

Fig. 6. Amino acid sequence of heavy chain of protein Eu. Positions in which amino acids differ in different heavy chains so far studied [11, 12] are indicated by dots. The V_H region extends at least to residue 115.

regions of κ chains fall into three subgroups that are non-allelic, i.e. each individual has at least three genes specifying the subgroups [14]. Individual proteins within each V region subgroup differ on the average in 10 positions and the substitutions may be accounted for by single base changes in the genetic code.

Although fewer examples of heavy chains have been studied [11, 15], they too have V and C regions (Fig. 6). The V_H region is approximately the same length as V_L and the evidence suggests that both V_H and V_L contribute to the antigen combining site [16]. *We thus have a molecular basis for the diversity requirement in the selective immune response :* the different amino

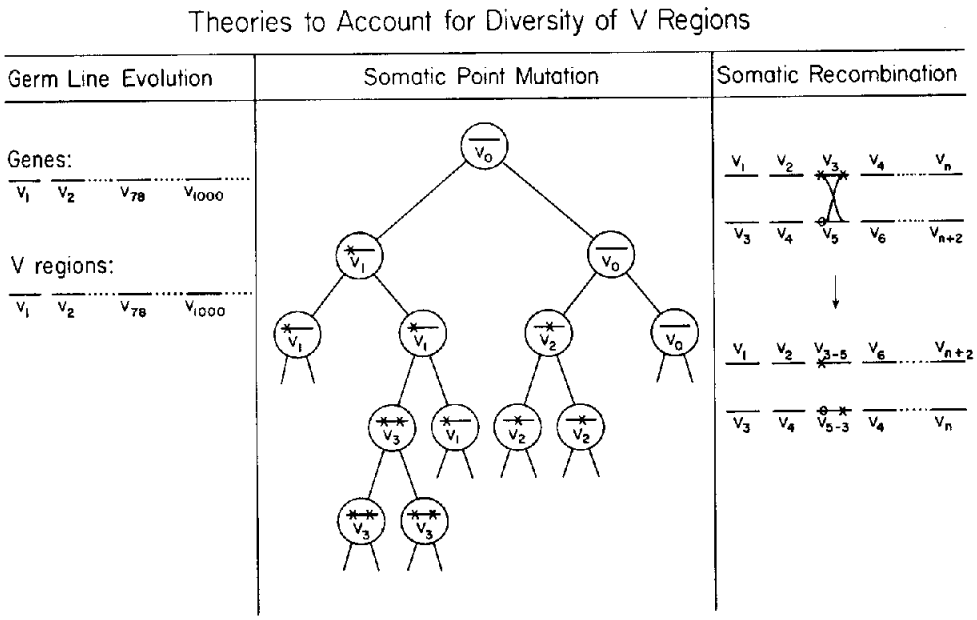

Fig. 7. Theories of diversity at the gene level. Germ line evolution : one gene for each V region. Somatic point mutation : hypermutation of single or few genes during development of organism with selection of products. x indicates different mutations in V gene. Somatic recombination : a small set of V genes with mutations selected for in evolution recombines in the lymphoid cell to give new combinations of sequences.

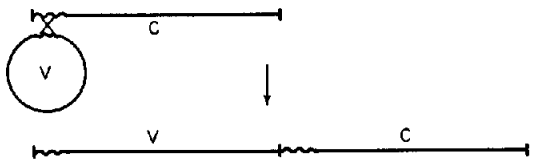

Fig. 8. A hypothetical model for translocation of V genes to C genes. Episomal V gene crosses over with one of the two C genes and the product is a single VC gene capable of being transcribed. Wavy lines indicate regions of integration and recombination.

acid sequences of V regions lead to different three-dimensional structures for the combining site. Moreover, there is a potentially enormous variety of sequences. We are left, however, with the problem of the genetic origin of this diversity. Three theories (see Fig. 7) have been proposed to account for diversity at the level of the gene : 1) There is a single V gene for each different V region and thus each organism contains an enormous number of V genes [17]. 2) There is one V gene for each type of chain and it undergoes somatic hypermutation. Following cell division, the mutants are selected for by an as yet unknown principle according to their capacity to make antibody molecules [18]. 3) There are a few V genes which have evolved to contain a selected set of mutations and these genes recombine in the precursors of antibody-forming cells to provide the diverse variants required [19]. A choice amongst these theories must await experiments to determine the number of genes specifying V regions.

Besides providing a molecular basis for the diversification requirement in a selective recognition system, studies of antibody structure have suggested ways in which the clonal nature of the system may have arisen [13]. As pointed out above, there are at least three V region genes and only one C

SEQUENCE HOMOLOGY IN EU VARIABLE REGIONS

EU V_L (RESIDUES 1 - 108)										ASP (1)	ILE	**GLN**	MET	THR	**GLN**	**SER**	PRO	SER	THR (10)
EU V_H (RESIDUES 1 - 114)										PCA	VAL	**GLN**	LEU	VAL	**GLN**	**SER**	GLY	–	ALA
LEU	SER	ALA	SER	VAL	**GLY**	ASP	ARG	**VAL**	THR (20)	ILE	THR	**CYS**	ARG	**ALA**	**SER**	GLN	SER	ILE	ASN (30)
GLU	VAL	LYS	LYS	PRO	**GLY**	SER	SER	**VAL**	LYS	VAL	SER	**CYS**	LYS	**ALA**	**SER**	GLY	GLY	THR	PHE
THR	–	–	TRP	LEU	ALA	**TRP**	TYR	GLN	**GLN**	LYS	**PRO** (40)	**GLY**	LYS	ALA	PRO	LYS	LEU	LEU	MET
SER	ARG	SER	ALA	ILE	ILE	**TRP**	VAL	ARG	**GLN**	ALA	**PRO**	**GLY**	GLN	GLY	LEU	GLU	TRP	MET	GLY
TYR	LYS (50)	ALA	SER	SER	–	LEU	GLU	SER	GLY	VAL	PRO	SER (60)	ARG	**PHE**	ILE	**GLY**	SER	GLY	SER
GLY	ILE	VAL	PRO	MET	PHE	GLY	PRO	PRO	ASN	TYR	ALA	GLN	LYS	**PHE**	GLN	**GLY**	–	ARG	VAL
GLY	THR	GLU (70)	PHE	THR	–	–	–	–	–	–	–	–	LEU	THR	ILE	**SER**	**SER**	**LEU**	GLN	PRO (80)
THR	ILE	THR	ALA	ASP	GLU	SER	THR	ASN	THR	ALA	TYR	MET	GLU	LEU	**SER**	**SER**	**LEU**	ARG	SER
ASP	**ASP**	PHE	**ALA**	THR	**TYR**	TYR	**CYS**	GLN	GLN (90)	–	**TYR**	ASN	SER	ASP	**SER**	LYS	MET	PHE	GLY
GLU	**ASP**	THR	**ALA**	PHE	**TYR**	PHE	**CYS**	ALA	GLY	GLY	**TYR**	GLY	ILE	TYR	**SER**	PRO	GLU	GLU	TYR
GLN (100)	**GLY**	THR	LYS	**VAL**	GLU	VAL	LYS	GLY											
ASN	**GLY**	GLY	LEU	**VAL**	THR														

Fig. 9. Homology in V regions of protein Eu. Identical residues in the amino acid sequences of V_L and V_H are shaded. Deletions indicated by dashes have been introduced to maximize the homology.

region gene for κ chains in each haploid set. A mechanism must exist [16] for transposing one of the V genes to the C gene so that a complete VC gene exists. One mechanism by which this may occur is shown in Figure 8. By translocation of a V gene episome followed by recombination, a VC gene with the appropriate start signals may be formed. This would effectively represent an irreversible differentiation step if it were followed by transcription and translation. It assures that the cell makes only one, or at most two, of the heavy or light chains from the vast set for which it has the genetic information. The commitment to one type of antibody guarantees that there will be

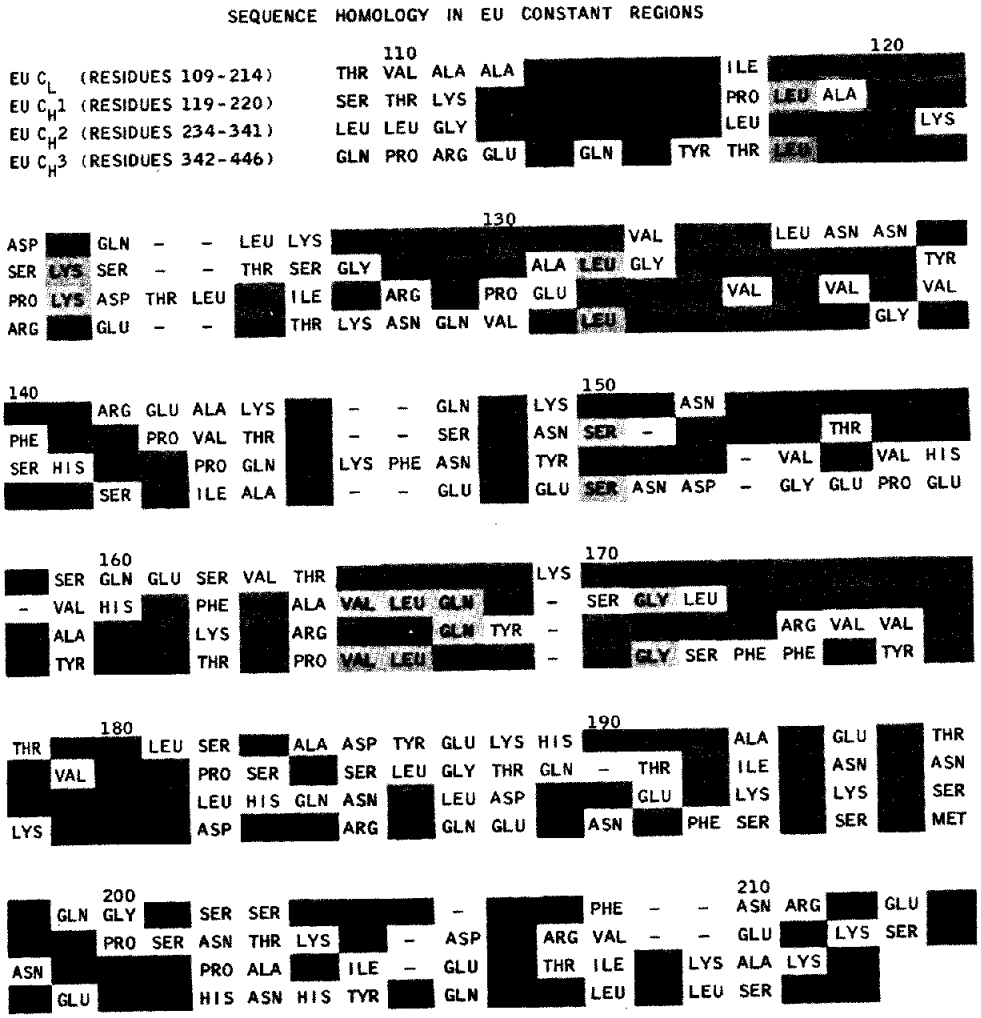

Fig. 10. Homology in C regions of protein Eu. Identical residues are darkly shaded. Dark and light shading are used to indicate identities which occur in pairs in the same positions.

no loss of specificity in the system and thus diversification is coupled to amplification (see Fig. 3) in an unequivocal fashion. Although the evidence on antibody structure is completely compatible with this hypothesis, it should be pointed out that it has not been proven.

An examination of the structure also provides evidence to support a scheme for the evolution of the antibody molecule to serve both antigen recognition functions and effector functions. Comparison (Fig. 9, Fig. 10) of the V_L, V_H, C_L and C_H regions shows that there are a number of homologies in the sequences. These are schematized in Figure 11. V_L and V_H regions are homologous to each other and C_L is homologous to three adjoining regions of C_H known as C_H1, C_H2, and C_H3. V regions do not show any obvious homology to C regions. We may modify the original suggestion of Hill *et*

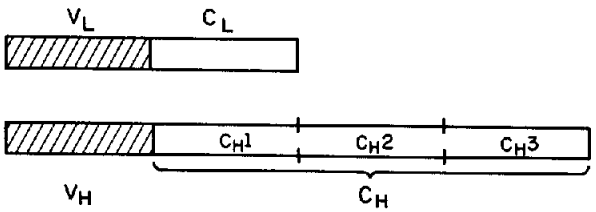

Fig. 11. Diagram summarizing internal homologies in the structure of γG1 immunoglobulin. Variable regions V_H and V_L are homologous. The constant region of the heavy chain (C_H) is divided into three regions, C_H1, C_H2, and C_H3, which are homologous to each other and to the C region of the light chain (C_L).

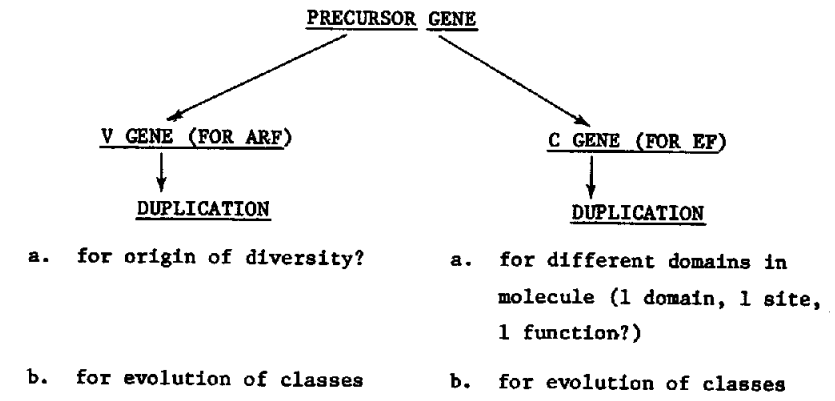

Fig. 12. Hypothetical scheme for evolution of antigen recognition functions (ARF) and effector functions (EF) of immunoglobulins.

al. [20] on the evolution of immunoglobulins in the fashion shown in Figure 12. According to this hypothesis, immunoglobulins arose by divergence of V and C genes, each capable of specifying a polypeptide chain of about 110 residues. Gene duplication and subsequent variation accounted for the development of the various kinds of V regions and C regions. Evolution of a translocation mechanism to join V and C resulted in a molecule with linear differentiation of function : V regions for ARF and C regions for EF.

This proposal is in accord with the covalent chemistry of the molecule. Thus there is a rotational symmetry axis in the Fc region which contains the homology regions C_H2 and C_H3 (see Fig. 4). Moreover, a pseudosymmetry axis may exist between V_H and V_L and C_L and C_H1. On these and other grounds, it has been proposed [13] that the immunoglobulin molecule is folded in compact domains each consisting of a homology region, containing at least one active site and serving a separate molecular function. As shown in Figure 13, V_H and V_L serve antigen recognition functions. According to the hypothesis, C_L and C_H1 may serve to stabilize the interaction of the V domains but may also play a role in triggering the cell to mature after encounter with the antigen. The C_H2 domains contain the carbohydrate which is required for export of the molecule from the cell [21]. C_H3 domains may contain sites for skin or complement fixation, but so far no function has been determined for these regions.

Verification of the domain hypothesis will rest on electron microscopic and X-ray crystallographic analysis of the three-dimensional structure of antibodies. This hypothesis and the evolutionary scheme discussed above suggest an overall sequence of events in evolution of the selective immune response. The first evolutionary event must have been the development of a diversifica-

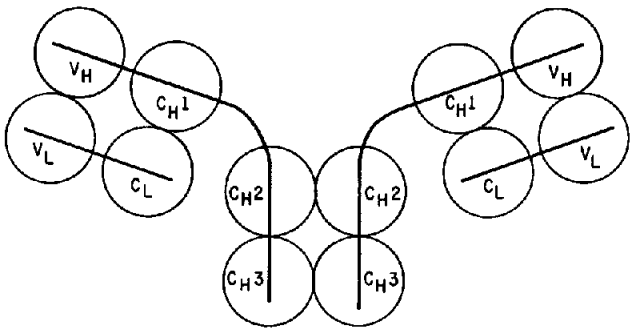

Fig. 13. The domain hypothesis. Homology regions (see Fig. 11) which constitute each domain are indicated. V_L, V_H - domains made up of variable homology regions. C_L, C_H1, C_H2, C_H3 - domains made up of constant homology regions. Within each of these groups, domains are assumed to have similar three-dimensional structure and each is assumed to contribute to an active site. The V domain sites contribute to antigen recognition functions and the C domain sites to effector functions.

tion mechanism to yield a sufficiently great variety of antibodies. These antibodies may have been fixed to cells similar to those concerned with delayed hypersensitivity reactions. Subsequent development of C genes, translocation mechanisms, and carbohydrate fixation would have allowed for export of antibodies and for development of effector functions, each mediated by a specific domain. This can account for the evolution of classes of antibodies other than the γG immunoglobulins. Furthermore, it would suggest that once diversification was present, the major evolutionary developments [22] to improve the selective response were focused on the amplification mechanism (see Fig. 3) and on the refinement of effector functions.

The purpose of this discussion has been to outline the minimal requirements for a selective recognition system, to show how these requirements might be related to our current knowledge of antibody structure, and to discuss a number of hypotheses on the origin of specificity and control in the immune response. It should be stressed that so far none of these hypotheses has been proven; nevertheless each has some evidence to support it. We may expect that a more detailed understanding of selective molecular recognition will come from studies of the three-dimensional structure of antibody molecules by X-ray crystallography, as well as from a more quantitative analysis of the population dynamics of antibody-forming cells.

Summary

Antibodies are the key molecules of immunity. They constitute a molecular recognition system and carry out two major functions in the immune response: 1) they bind to a great variety of sterically different antigen molecules (antigen recognition function or ARF), and 2) they mediate essential immune reactions within the orgnaism such as complement fixation (effector functions or EF). The ARF is *selective,* i.e. the information for synthesizing antibodies specific for a large range of chemically different antigens *already* resides in the organism before exposure to those antigens. Particular antigens serve only to stimulate (and thereby select) those antibody-forming cells which synthesize antibodies having appropriately complementary binding sites. This requires that the number of sites must greatly exceed the number of different antigens. Furthermore, there must be an exceedingly efficient system for amplifying the production of specific antibodies following stimulation by injection of a particular antigen.

It appears that these requirements are met by several mechanisms, the details of which are incompletely understood. The antibody-forming system can produce an enormous number of different antibodies with diverse amino acid sequences and therefore with antigen binding sites of different shape. Each cell is committed to producing only one of the many kinds of antibodies.

Following encounter between the antigen and the " correct " cell, the cell is stimulated to mature and divide, and its progeny produce greatly increased amounts of antibodies of the same kind. Thus, according to this view, the selective response is clonal.

Studies on the molecular structure of antibodies or immunoglobulins have been useful in analyzing some of the mechanisms of the selective immune response. The covalent structure of an entire γG immunoglobulin (molecular weight 150,000) has recently been completed and compared with portions of other immunoglobulin molecules analyzed by other workers. The molecule consists of a variable or V region and a constant or C region. The amino acid sequence of V regions differs from molecule to molecule whereas the sequence of C regions is relatively invariant. The mechanism by which diversification of amino acid sequence is achieved in antibody V regions has not been established, but several theories on the origin of diversity have been proposed and are reviewed.

The data on amino acid sequence corroborate the hypothesis that the antibody molecule evolved from two genes, V and C, each of which underwent successive duplication. The structure of the γG immunoglobulin molecule shows a linear periodic arrangement which reflects its origin by gene duplication. In accord with this order and the symmetry properties of the structure, it is suggested that the molecule is arranged in successive compact domains organized about axes of symmetry and pseudosymmetry. According to this proposal, domains of V regions mediate antigen recognition and successive domains of the C regions mediate different effector functions.

Previous evidence has suggested that each polypeptide chain is specified by a V and a C gene which may be translocated in the lymphoid cell to form a single VC gene. This hypothesis can account for the clonal nature of the selective response, for although each cell may have information for many V sequences, it has at most only two C genes for each chain. Translocation would commit the cell to the expression of the smallest possible number of antibody chains and help to maintain the specificity and efficiency of the selective immune response.

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DISCUSSIONS

E. MARGOLIASH : I have a pair of suggestions, we discussed some of them, and I think it might be worth bringing them out. The classical way in which the evolution of the chains for the gamma globuline has been considered is a business of duplicating a segment of something like 110 to 120 residues long. In point of fact as you know, it's beginning to look more and more as if a molecule which is much older than gamma globuline has essentially the same structure, myosin : namely two light chain, and an extremely long heavy chain, two very long heavy chains giving an overall picture which is extremely similar to that of gamma globuline including a little bit of amino acid sequence, which might indicate that in fact, the gamma globulines arose from the myosines by deletion of a long section of the heavy chains and not the other way around. There are in fact several sizes of heavy chains and one might consider that they arose independently by removing different legs of the tail of the heavy chain of the myosin-like molecule. Myosins of course go back all the way into taxonomy, way back whereas gamma globulines are relatively recent protein of vertebrates. Only. They are both made by the same cells - similar, embryologically similar cells. They both arise from the mesoderm. This is one thing.

Another suggestion I had, I would like very much to have your opinion on : is it possible to consider the binding sites in the anti-body as a certain sphere which you know we estimate at about 5 angström in radius and 10 angström in diameter, and that therefore, the ways in which one can dispose masses and charges from a protein in such a sphere, is really rather limited. So that in the evolution in the various series, or possible series of the evolution of the V genes, of the evolution of the variable regions, nothing would ever be wrong. Everyone that can possibly be made, would correspond to something. And this would eliminate a very large number of the troubles that you have with the various theories of the evolution.

G.M. EDELMAN : Yes. I have nothing to say about your other comments but I'd like to speak about this last one.

The fact is that affinity-labeling studies show that there is a tyrosyl residue right near the disulphide bond of the V region. That seems to be a relatively constant feature. Singer, who has found this, suggests that in fact the site is located in the vicinity. It does not drift around, it is located in a very specific way, and has some constant features.

The second point I should like to make is that, clearly, from the sequence itself, we see an enormous amount of selection, just as much selection as you see for cytochrome C. If we entertained your idea, I think that we would have to say that there must be a very special structure in the site because we just don't see random replacements of amino acids. We see just as limited a number and just about the same proportion of transitions and transversions that you see.