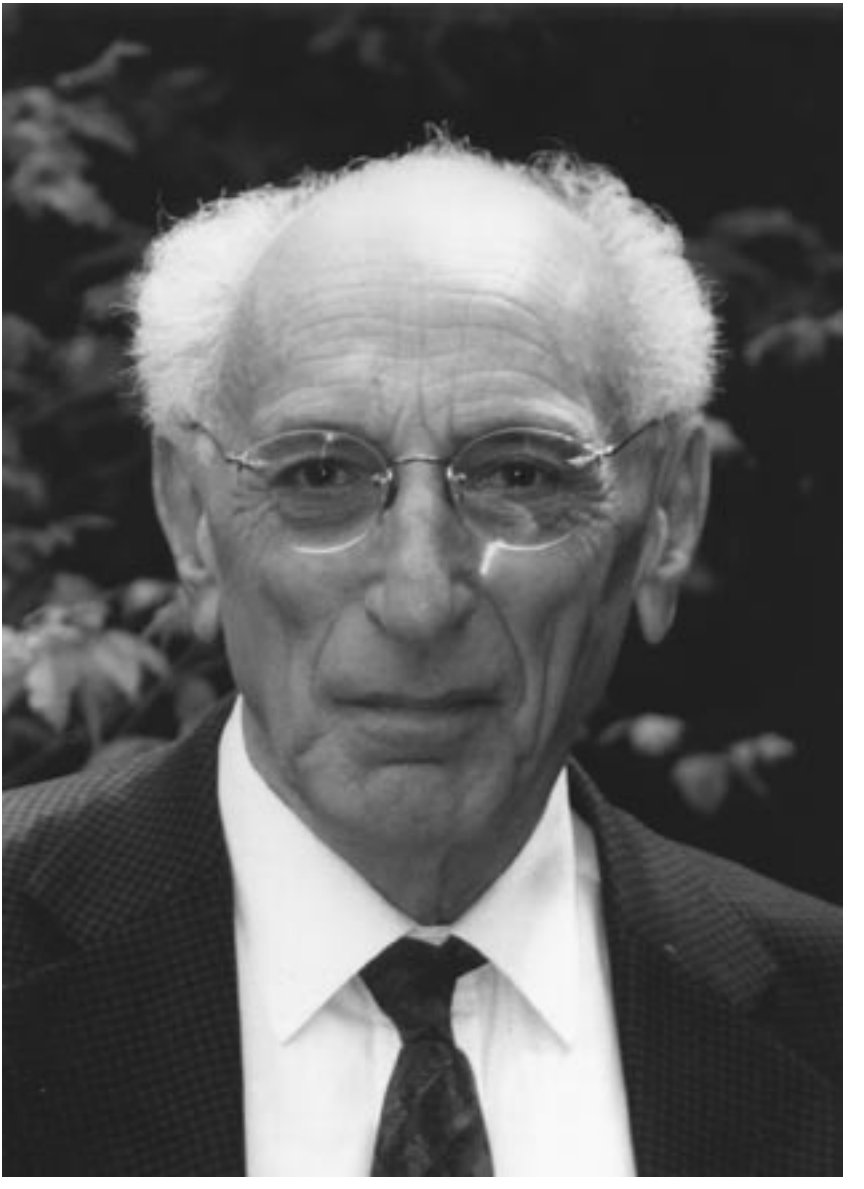

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TARGETS OF DRUG ACTION

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■ **Abstract** This article considers early work from the author's laboratory on muscarinic receptor specificity, subtypes, and conformational variability, with the use of nuclear magnetic resonance in pharmacology and the conformational variants of dihydrofolate reductase and general questions of receptors. It also considers some current approaches to drug development and receptor function, particularly as influenced by increasing knowledge of three-dimensional structure of receptors.

A CHANGE OF PLANS

Like many pharmacologists, my original training was in medicine, and I fully expected to spend my career in clinical work, as a pediatrician. However, immediately after graduation in London, I was seriously ill and one of my frequent visitors was my professor of physiology, Samson Wright. He persuaded me not to go straight into clinical work on recovery but to spend some time in a laboratory. What about joining the new pharmacology department at the Middlesex Hospital Medical School that Cyril Keele was just establishing? he asked. I said that I would, for a year only, but then I got hooked. I began attending the monthly meetings of the Physiological Society (in those days there was no separate pharmacological society), and the major topics presented were concerned with the growing evidence that the role of acetylcholine as a transmitter could be extended from the parasympathetic postganglionic endings to the sympathetic ganglia and the neuromuscular junction. The experimental evidence in favor was developed by WS Feldberg, GL Brown, Bernard Katz, and FC MacIntosh under the genial, but critical approval of our doyen, Sir Henry Dale. These developments were strenuously opposed by JC Eccles, who held to the all electrical theory of synaptic transmission and that all the acetylcholine effects people were looking at were ''epiphenomena.'' This in no way discomforted Feldberg, who wrote of the extension of the theory to the central nervous system with great confidence: ''The present position of the theory of acetylcholine as central transmitter is all but settled.'' Later he added a bit of caution: ''perhaps not the universal central transmitter.'' This all made for a very exciting time, and quite naturally I found myself

working on cholinergic problems. This was 1945, when news of the wartime work on organophosphorus anticholinesterase compounds became public. They were a fascinating series of compounds producing irreversible inhibition with apparently great specificity, and so, like Avram Goldstein (1), I began to study them. Like Avram, I needed to learn the mysteries of the Warburg and also to tackle kinetic problems. It was proved that the action was due to dialkylphosphorylation of the enzyme at the active site responsible for substrate hydrolysis, and that the inhibition was not totally irreversible but that the stability of the phosphorylation depended on the attached groups. Subsequent evidence obtained by others showed that the phosphorylation was on a serine in the active site. I regret that we failed to realize that the very slow kinetics of eserine and prostigmine inhibition also indicated an acylation of the enzyme.

One day, a colleague in the biochemistry department, Frank Dickens, came by with a problem. He had seen a paper by Torda and Wolff that claimed that botulinum toxin inhibited the synthesis of acetylcholine by cholineacetylase, which excited him, and he and a postdoc, Leonard Zatman, had acquired a grant to study it in detail. They had received a sample of highly purified toxin from the chemical warfare group at Porton, but alas, this sample of toxin had absolutely no effect on acetylcholine synthesis. Evidently, the effect described by the previous authors had been due to impurities. So how did botulinum work and could we help? The isolated phrenic nerve-diaphragm preparation had recently been introduced by Edith Bülbring, so we tried the toxin on that and it produced very reproducible neuromuscular block, which we could analyze. The block was not at all like that produced by curare. The muscle remained completely sensitive to the close intravascular injection of acetylcholine. We found a way of measuring the release of acetylcholine when the phrenic nerve was stimulated and discovered that its release was greatly reduced. Thus, botulinum acted on the release process presynaptically and synthesis was quite unaffected (1a).

MOVING TO MONTREAL

One evening after visiting my wife in hospital, where she had just given birth to our daughter, I got on a bus and encountered FC (Hank) McIntosh, who finding where I had just been, said, ''Come home and wet the baby's head.'' In the course of the evening he told me that he had accepted the chair of physiology at McGill University in Montreal. ''What about coming too?'' he asked. Thus, shortly after the botulinum work was completed, I moved with him to McGill, where one of my next pharmacological adventures occurred. This adventure concerned the action of acetylcholine on the auricle examined with intracellular electrodes and was carried out with Kathleen Terroux. Acetylcholine increased the resting potential and radically reduced the duration of the action potential by increasing the rate of depolarization. The potassium dependence of the resting potential was also increased. We concluded that the effects on the electrical properties could all be attributed to an increase in potassium permeability, but we had no idea how the

negative inotropic action was produced. This was the first demonstration of the mechanism of an inhibitory action of acetylcholine (2). Further development had to be left to others because I succumbed to a recurrence of my illness. When I returned to work, it seemed too disheartening to try and catch up, so I started a new line of work to try and disentangle the effects of parasympathetic stimulation on secretion in the salivary glands. The effects were dramatic enough: The maximum rate of saliva secretion per minute could exceed the weight of the gland. This not being the place to discuss details of the complex processes that have been uncovered in the secretion of water and electrolytes, suffice it to say that it is a sort of neurogenically controlled ''kidney'' where the fluid is produced by the acini and modified as it travels down the ducts. However, one aspect of the action of acetylcholine was novel. I found that some hydrophilic nonelectrolytes that could not enter the salivary gland cells passed into saliva in rather high concentration, which could best be explained if acetylcholine had opened intercellular channels (3). Later, Konrad Martin and I looked at the influence of sympathetic stimulation and found that this effect was very dramatic—even large nonelectrolytes such as sucrose were secreted—and so the possibility of a paracellular path could not be ignored (4). It has subsequently appeared from other work that a feature of the secretory process is a paracellular equilibration that essentially equilibrates the composition of the primary secretion produced by the acini with that of the plasma. The nature of the receptor-effector system responsible seems poorly defined, but norepinephrine opens up larger channels than acetylcholine does.

In 1957 I had the good fortune to spend the summer at Woods Hole working with that master neurophysiologist, Stephen Kuffler, on the (noncholinergic) nerves that inhibit transmission at the lobster neuromuscular junction (5). We made extracts of lobster nerve cord and muscle and found activity on muscle and the heart. To separate activities, we used paper chromatography with rather crude paper (all the large sheets of filter paper from the MBL stores had yellow marks on them, which on enquiry we found were due to the stores having been flooded the previous winter). Nevertheless, the separation worked all right, and it looked as though the activity might be due to the amino acids in the extracts, notably glutamate and aspartate; these had strong stimulatory activity on the lobster heart (6) and gaba was inhibitory. I should add that when I made alcoholic extracts of lobster muscle, the residual chunks of meat were very popular!

On my return to Montreal, I also ran a research unit at the Montreal General Hospital and had a chance to do some human pharmacology as well as some pharmacokinetics on the folic acid system.

PROFESSOR OF PHARMACOLOGY IN CAMBRIDGE

By 1962, it was becoming clear that the receptor concept was the basis of most, if not all, drug action and that most receptors were likely to be the site of action of endogenous regulators, mostly still unknown and whose role was yet to be

defined. So far only the tip of the iceberg had been uncovered. Much of the future of pharmacology thus would be concerned with four problems: (*a*) defining the receptors, including isolating and characterizing them; (*b*) finding the endogenous regulators; (*c*) developing drug analogues; and (*d*) trying to understand the basis of the specificity of drugs for receptors. I decided that for the near future I wanted to concentrate on the fourth of these objectives, and I drew up a theoretical analysis that provided a protocol of what details we needed to find out about drug-receptor complexes (7). In order to develop such a program, it was necessary to select systems suitable for this kind of evaluation (it will not be surprising that the theme of acetylcholine keeps reappearing!).

My colleagues, Rod King and Palmer Taylor, and I decided first to examine the structure-activity of the aromatic sulphonamides as inhibitors of carbonic anhydrase. Because the binding constant to a site represents the ratio between the association and dissociation constants for a ligand, we thought that examining the rate constants by fast kinetics might give some useful clues. We started with two expectations: that the strength of binding would be mainly reflected in the stability of enzyme-inhibitor complex, and hence in its dissociation rate, and that the association rate would be dependent primarily on the rate of diffusional access to the binding site and would not change much between different sulphonamides. To our surprise, this was not the case. The binding constant correlated well with the association rate and the connection with the dissociation rate was clearly both smaller and less consistent (8). This was seen particularly clearly in a simple homologous series of 4-alkyl benzene sulphonamides, where the binding increased steadily up to C_5. There was only a small change in the off-rate, but the increased binding was reflected mainly in an increase in the association rate, which was in any case far below the limit set by diffusional access. When we looked at a similar series of 3-alkyl esters of benzenesulphonamide, the increased binding showed no dependence at all on the off-rate (9). By examining the pH and ionic strength dependence, as well as the binding to the apoenzyme and cobalt enzyme, we produced evidence that this unexpected behavior could be attributed to the formation of an intermediate hydrophobic complex (not complexed with the zinc in the active site) whose concentration was the rate-limiting step, and which was followed by a unimolecular transformation into the final zinc-coordinated complex. Changes in ligand structure were concerned mainly with determining the concentration of the intermediate complex, which was reflected in the rate of formation of the final complex, and that is why the stability of the complex was so dependent on the on-rate (10). It seems likely that multistep processes in forming drug receptor complexes are common. In this instance, no significant conformational change in the enzyme was involved, but as discussed below, in the formation of many drug complexes conformational changes are present.

We once thought receptors were simple. I began to have doubts when Laurence Spero and I developed an elegant method for following the efflux of potassium from intestinal smooth muscle in response to muscarinic agonists (11). To our suprise, we found that the dose-response relations for this were different from

those for muscle contraction. We then played with the possibility that there were distinct receptors for the two responses, but the evidence did not support this. Rather, it led us to conclude that the receptor could be coupled to different responses in different ways (12). With Nigel Birdsall, John Young, Robin Hiley, and Ed Hulme, we went on to make precise measurements of the binding of ligands to muscarinic receptors, initially in the cerebral cortex. The binding of antagonists seemed to obey simple mass action rules and to accord very well with the affinities found by inhibition of contractile responses in smooth muscle (13). The trouble began when we looked at the binding of agonists (14), which was not simple but corresponded to at least two binding sites of very different magnitude. Later we found we could identify three such components, the proportions of which seemed the same for all full agonists, but which were different in different areas of the brain (15). These different forms of the receptor seemed stable because when we inactivated one form, the residual forms did not reestablish the proportions. On the other hand, we could convert the forms with the highest affinity into the lower-affinity forms by simply raising the ionic strength (16). When cardiac receptors were examined, the situation seemed similar, but it was not just high-ionic strength that could convert the higher- into the lower-affinity forms. This change was also dramatically produced by GTP, which converted virtually all of the highest-affinity form into the lowest-affinity form (17)—a further hint at the physiological significance of the multiple forms.

At this point, Rudi Hammer appeared on the scene bearing a new drug, pirenzipine, which in animal and human studies showed selectivity notably toward gastric secretion. Lo and behold, it was an antagonist, which by a factor of approximately 20 was more active against cortical than smooth muscle receptors, and even more surprising, some of the binding curves, those for cortex, hippocampus, and submandibular gland, did not have unitary slopes (18). Our analysis led to the conclusion that we were seeing the presence of at least three subtypes of the receptor; subsequently, a few other ligands were found that also showed discrimination of this kind. It was not long before the cloning of receptors got underway, and soon there was clear evidence of at least five subtypes and of the now-familiar situation found with other receptors: the existence of many subtypes with widely different amino acid sequences. It remains interesting that despite the very different sequences in the subtypes, so few ligands discriminate between them, a fact that needs to be borne in mind in elucidating details of receptor binding sites.

I deal with just one other aspect of muscarinic receptors. In 1951, Riker and Wescoe reported that the neuromuscular blocker, gallamine, acted on cardiac muscarinic receptors, an action shown by Clark and Mitchelson not to be competitive. Stockton, Birdsall, Hulme, and I reexamined the effects of this substance in the binding assay. We found that gallamine reacted with the muscarinic receptors in heart selectively, and although it was not competitive, it acted allosterically, reducing the affinity of both antagonists and agonists, by as much as 250-fold in the case of oxotremorine-M. It also dramatically slowed the reaction of antagonists with the receptor, a reliable indicator of allostery. Evidently, gallamine interacts

with a second site in the receptor, distinct from that for the agonists or antagonists producing a changed conformation, which is reflected in different degrees of modification of binding for each ligand; i.e. the new conformation has a different structure activity profile (19). Some other substances have subsequently been found to have this same kind of action. It is interesting that gallamine also acts allosterically on cholinesterase.

The lessons to be drawn from the multiple states in which muscarinic receptors can exist are of general importance for the understanding of drug action. It has long been assumed that the interaction of agonists somehow changed the receptor to bring it into an active state, but the evidence from the kind of results recounted above show that a variety of conformational states are accessible to the receptor, depending both on other components of the receptor complex and on the nature of the drugs reacting with it. Such characteristics have been studied in most detail in the receptors coupled to ion channels, notably bacteriorhodopsin and the nicotinic receptor, which leave no doubt about the complexity of the transient and more-stable states that can exist. It has been pointed out on a number of occasions that the states of a receptor as seen by agonists and antagonists respond differentially to such features of drug structure as stereochemistry, the size and polarity of substituents, and so on. Because of the conformational changes, the ''face'' of the receptor presented to the ligand is altered, maybe radically, and this has importance in considerations of drug design.

HARVARD

One day in Cambridge I met Oleg Jardetzky, who was on a sabbatical in the biochemistry department, and he interested me in the possibility of using nuclear magnetic resonance (NMR) in pharmacology. The result was that I spent the summer of 1966 in Oleg's lab in the pharmacology department at Harvard. I arrived with an antibody against phenoxycholine, which bound acetylcholine, and with my postdoc, Jim Metcalfe. The NMR machine we used was an early unlocked type, which needed a lot of magnet adjustment and did not have perfect voltage stabilization. This meant that before starting a run, we had to find out whether there was a ballgame going on in Boston. If there was, it precluded an experiment because the mains was too unstable! However, we were able to show that the freedom of motion of tetramethylammonium was reduced by the antibody and that there were differential effects on the motion of groups in choline esters and ethers (20).

The considerable range of possibilities for the study of pharmacological problems by NMR and other physical techniques led to the British Medical Research Council establishing a Molecular Pharmacology Research Unit in the Department of Pharmacology at Cambridge, which provided excellent facilities and staff positions; notably, it enabled us to recruit an NMR specialist, Jim Feeney, from Varian Associates. So what did we find out by NMR? We started to look at conformation

problems. We examined the motional characteristics first of small molecules, such as alkyl ammoniums. We were able to show that most of their motion in solution was due to a tumbling of the whole molecule rather than to rotation around bonds. We also showed that such small peptides as TRF, LHRF, and gastrin tetrapeptide were random coils without any evidence of tertiary structure (21). In the normal state, with intact SS bonds, the structures of vasopressin and oxytocin resembled each other, with restricted motional possibilities in the ring, and showing no evidence of either hydrogen bonding or the tail peptide being folded over the ring, as had previously been suggested. When the disulphide was reduced, they showed the same flexibility as the other linear peptides (22). These observations led to a discussion of whether flexible molecules bind to their receptors, by selection from the population of conformers or by conformational adaptation of the first weakly bound complex, the so-called zipper mechanism (23). The latter mechanism implies that a weak interaction of part of the molecule with the receptor occurs initially, with the full interaction developing as a result of rotation of the bonds in the nonbound part of the molecule. This kind of mechanism has been considered extensively for the problem of interaction of DNA in forming the complementary double helix.

A study with Phil Seeman and Jim Metcalfe of the interaction of anaesthetics with cell membranes showed that the mobility of anesthetics was greatly reduced by the membrane and that they were almost certainly confined to the lipid phase of the membrane. As the concentration of anaesthetic increased, the mobility increased, reflecting an increased fluidity of the membrane lipid, and only when the concentration reached levels that were lytic did any evidence of binding to protein emerge (24).

We decided that a combined kinetic and NMR study of a well-defined system of a protein and ligands would be interesting, and our choice fell on bacterial dihydrofolate reductase (from *Lactobacillus casei*), a relatively small, single-subunit enzyme of great importance in relation to chemotherapy. Despite its small size, it has binding sites for rather large molecules, such as the coenzyme NADP (H), and for folates, as well as for such inhibitors as methotrexate and trimethoprim. The detailed studies that followed could not have been achieved without a strong team, which included Jim Feeney, Gordon Roberts, and Berry Birdsall. Binding studies soon showed the complexities we could anticipate. The binding of NADPH together with methotrexate was highly cooperative, increasing by over 700-fold, but on the other hand it was equally negatively cooperative with folinic acid, whose binding was reduced 500-fold, so that the binding of NADPH was changed by a factor of 3.5×10^5 by the binding of these second ligands (25, 26). Another interesting finding came from the realization that the binding site for methotrexate (or folate) was so extensive that we had to consider the possibility it might be occupied by two fragments of the ligand simultaneously. We found that p-aminoglutamate (PABG) bound and so did 2,4-diaminopyrimidine (DAP), and that they could bind simultaneously and did so cooperatively. When we looked at a series of N-alkyl derivatives of PABG, the binding increased with

chain length in the binary complex, by two orders of magnitude for the hexyl derivative, but when DAP was also bound the effect of chain extension was completely lost (27). We soon found that the complex dependence of binding on the structure of the ligands required the postulation of multiple conformations. This is where NMR showed one of its strengths. Using proton NMR, one could see in the complex of the enzyme with trimethoprim and NADP (*a*) two sets of proton resonances from the nicotinamide end of the NADP—one set shifted upfield and the other shifted downfield—and (*b*) that two of the enzyme histidines were doublets. With ^{31}P NMR, one could see two sets of resonances for the pyrophosphates. It was abundantly clear that two conformations of the enzyme were present in roughly equal amounts, and they interconverted relatively slowly, so that distinct spectroscopic differences could be observed for both the enzyme and the coenzyme; the binding curve had shown only a simple, single binding constant (28). In another study, using NADP on whose carbonyl group ^{13}C replaced the normal ^{12}C, we saw by ^{13}C NMR that in the ternary complex with folate, the carbonyl group was present in three distinct, coexisting conformations whose relative abundance was altered by pH (29); evidence for this had also been found by fast kinetics. How exciting it was to actually have direct evidence of conformational populations, even though binding studies had left no doubt of their existence. In some complexes where the conformation was predominantly in one form, NMR enabled us to pick out aspects of the binding of ligands that put a marker on the conformations involved, but it did not give us a global picture of the binding site or of the overall conformation of the protein. Of course, X-ray structures can do that, but you need structures for many complexes to be able to interpret fully the structure activity problems.

I have said enough, perhaps too much, about how questions of the conformation of receptors absorbed me during this time. To quote from a review I presented in 1981, ''drugs essentially interact with regulatory systems and the biological machinery for regulation is predominantly through conformation control.''

I should not forget to tell you that in the middle of all this I moved to become the Director of the National Institute for Medical Research in Mill Hill, London in 1971 and I am happy to say that the Molecular Pharmacology group came with me and the work went on.

RETIREMENT

In 1982 I retired from Mill Hill. I went back to live in Cambridge and became the head of Darwin, a graduate college in Cambridge University. I also became responsible for the international activities of the Royal Society, as Foreign Secretary. These activities took up so much of my time and energies that it became impractical to continue in the laboratory. Thus, in 1984, my life as an experimental scientist came to an end. I also had another new interest in the creation of a

European academy of science, the Academia Europaea, which was inaugurated in 1988. For these reasons, what I have written here is essentially ancient history. Any account of the later developments in the fields I have covered must be left to those who are still busy there.

However, I take this opportunity to stand back and look at some of the things happening in pharmacology and to hazard a guess or two as to where it is going. Pharmacology has always been closely interdependent with its related subjects—physiology, biochemistry, pathology, and clinical science—and together with all of these sciences has been hugely influenced by the development of molecular biology. Among other things, this has meant a much greater dependence on instrumentation and the need for larger research teams. The trend is likely to increase further, but it is unlikely to rival what has happened in physics. Academic pharmacology is mainly concerned with understanding how existing drugs act. I return to the question of drugs intervening in regulatory systems. A therapeutic action on a regulatory system implies either that we may be dealing with a disordered regulatory system and that what we seek to achieve with drug action is to restore some component to within normal limits, or alternatively that a therapeutic result can be obtained by shifting the normal level of regulation. A major problem is that because of the parsimony of biological structure, the elements of the system we want to influence are likely to be used in other systems that we do not wish to perturb.

Recent research has yielded a great deal of information about how receptors work and, in some cases, details of their structure. It is now clear that the existence of receptor subtypes is a general condition that offers a prospect of finer discrimination, including differentiation of the effector systems to which they are coupled and which could lead to more selective action of drugs. However, finding drugs selective for individual subtypes is far from a simple matter. Is the effort worth it and how far will this deal with undesirable side actions? A promising way of finding out is to use selective mutagenesis to knock out single subtypes and to establish both what physiological changes result and also the change in reaction to the available nonselective drugs for the sytstem. A recent report on knockout of muscarinic M2 receptors showed the panoply of actions that this subtype is involved in (30). It was no surprise that the delineated actions of the M2 receptor were multiple, involving not only the heart but several central nervous system actions too. Studies of this sort would be especially valuable if carried out over the lifetime and generations of test species. The limitations of simply seeking specificity at the receptor level are highlighted. A really selective M2 antagonist might be a better drug, but it would not attain the ideal of producing only a single physiological change. Further selectivity would have to be dependent on other features of the global activity, such as the criticality of the regulatory system in the various physiological systems, and how the selectivity is influenced by pharmacokinetics and by promoters such as cytokines (31). However, it is to be expected that many knockout studies will be forthcoming and will help to define with greater precision the range of drug actions. Interference with regulatory

systems invokes compensatory changes in second-order systems such as receptor down-regulation and changes in transcription, and there is also clear evidence that interference in the regulation of one receptor may secondarily interact with other related, and also not obviously related, systems (32, 33). These are ancillary targets that could be exploited in combination.

A perpetual problem in therapeutics has been those individuals who show atypical sensitivity to a drug or who develop side effects. Many of these cases are likely to be related to genetic differences. Recent studies of single nucleotide polymorphisms in β-adrenergic receptors provide the beginning for understanding some of them and indeed for describing pathology related to variant receptors (34). The question is global in its scope and is likely to define some uncommon new pathological states, but it could have a practical value in therapeutics in the future. A consortium of major drug companies has recently set up a project to map these polymorphisms, with the objective of minimizing side effects by identifying those that might affect drug responses; it is reported that at least one firm has already incorporated a search for polymorphisms in its drug discovery program (35). It is not likely to be long before quick tests for individual gene sequences may be so generally available that it will be feasible to pretest patients before giving them therapy. The mechanisms through which drug receptors are localized to particular types of cells and within cells is also just beginning to be explored (36). Mislocalization is another pathological possibility that could have interesting consequences, and therapeutic alteration to the balance of locations could be another basis for drug action. Developments in cell biology are uncovering an abundance of novel aspects of cell and tissue regulation. Some may reveal possible new targets for drug intervention, which may turn out to have therapeutic implications or to improve our understanding of toxicity. The control of blood vessel growth as an anticancer therapy is one such. The research in this area is so diverse and is changing so rapidly that it is not practical to go into detail here.

In the past, new drug development has followed several paths: the selection of some organ system or disease on which new synthetic compounds can be tested; the examination of the spectrum of activity of a novel compound, either synthesized or found naturally in plants or microorganisms; or the serendipitous observation of an extraneous activity. The latter was, for example, the origin of the antidepressants, following the observation that tuberculosis patients receiving isoniazid or iproniazid experienced a sense of well-being greater than that accounted for by the improvement in their physical state. An interesting recent example has appeared in the attempt to overcome bacterial resistance to vancomycin by chemical modification: Aryl substitution on one of the sugars revealed a different mechanism of action, which could well be the starting point for a new group of antibacterials (37).

When a compound with a novel action has been identified, its subsequent development into an effective agent that ends up in our therapeutics is by no means trivial, and some current developments are directed at improving and accel-

erating at least the discovery process. These include high-speed, semiautomatic testing of large numbers of compounds on isolated systems, mainly using receptor binding or enzyme activity and combinatorial synthesis to produce large numbers of candidate compounds to put into the high-speed tests. The latter is particularly attractive in peptide synthesis because the synthetic steps are merely repetitive. A good example of both these developments was the search for an analogue of enkephalin composed of **d**-amino acids. An automated synthesis yielded a set of 52 million acetylhexapeptides with practically all the possible sequences. These were tested in groups for μ-receptor activity on rat brain homogenates by the displacement of labeled DAMGO from the receptor. Iterative selection from the active groups subsequently led to the identification of the best sequence, which bound with the respectable value of 18 nM (38). Combinatorial methods can also be used for other syntheses and could, for instance, produce compounds in which some basic pharmacophor is varied by many substituents. Commercial packages for exploiting these methods are now readily available.

Although these enhanced trial-and-error methods will continue to be exploited, the hope of designing drugs in a more rational way is fundamental. In principle, this is available when the structure of the receptor is known and the area of the surface with which a drug interacts has been identified. Most drug binding sites are on proteins, and the three-dimensional structure of proteins is being determined at an astonishing rate by X-ray diffraction, by electron microscopy, and by NMR, so that several thousand are now known and recorded in the Protein Database. They include a number of important drug sites, such as dihydrofolate reductase and HIV proteases, for which the structure of complexes with inhibitors is also available. These structures give invaluable information about the three-dimensional structure of the effective binding site for that ligand and they yield details of the interatomic interactions involved. There are few surprises about the kinds of interactions identified, except for the discovery that bound water may be an important bridge. Calculations of the binding energy can be made using standard methods for electrostatic, hydrogen-bond, and dispersion interactions. Increasing levels of sophistication can be employed, and in some, though by no means all, cases they can give an encouraging order of magnitude agreement with the experimental figures (39); the energy calculation is very sensitive to the precision with which the groups are apposed.

The attempt can also be made to fit other active molecules presumed to be binding to the same site in the absence of an experimental structure for the complex. This uses computer methods that explore all the mutual dispositions of the ligand with the receptor, that calculate the free energy, and that select the configuration(s) giving the highest binding energy; many programs will also cover the range of conformations available to the ligand in binding if it is not a rigid molecule (40). When the binding of trimethoprim and methotrexate for DHFR was examined in this way, it was found that the optimal fit to the binding site was for conformations different from those found in the crystalline state (41); this was already known for methotrexate from the X-ray data. Note that there will be

penalty in energy terms in such a selection, and this is usually ignored in the calculations. Now, the binding area for trimethoprim is smaller than what is known from the X-ray structure to be available for methotrexate, which interacts with an additional area. Thus, trimethoprim by no means exploits all the possibilities of binding to the site, and it is not surprising that when trimethoprim was extended with a carboxylic chain related to the glutamate end of methotrexate, the affinity was increased by up to three orders (42). You will notice how this refers back to the binding of fragments of methotrexate discussed earlier. It means also that if the structure of the binding site is known, a wider range of possible binding subsites may be revealed and alternative combinations of subsites may be identifiable, leading to the proposal of novel drug structures. More generally, it means that visualization of the whole potential binding site as a three-dimensional surface on which hydrophilic and charged areas are displayed is an open playing field on which to start afresh to find molecules that give the optimal match to this surface. Several strategies are available for seeking such a molecule. A structure database can be accessed and used as a source of selected compounds to be tested against the whole site. An alternative approach is to visualize subsites one at a time, to find molecular fragments to fit them, and then to generate a complete molecule by filling in connections between the fragments.

So far in this account we have assumed that the conformation of the receptor site is invariant, but some of the amino acid side chains and even some of the peptide chains may be mobile. The fit to the receptor will then be less precisely prescribed. This has already been noted in inhibitors of HIV protease and adds a complication, but it provides an extra measure of flexibility in choice of structures (43); there is no intrinsic reason why allowance for this cannot be built into fitting programs.

Major conformational changes of the sort we have discussed for antagonist-agonist pairs are a different matter. This is clearly a problem. It might be anticipated that the conformation found in the unoccupied (ground state) would have a good prospect of matching antagonists, although there is no reason to assume that all antagonists combine with the ground state (vide DHFR inhibitors). For agonists the situation is intrinsically more complex, because by definition they are binding to a changed conformation. There remains the possibility that only part of the binding site is transformed in the active conformation and that subsites might therefore still provide guides to lead compounds from which conventional development techniques could be used. Of course, a natural regulator molecule for the site will usually be known and may provide a lead, and another strategy is considered below.

There will be situations where the three-dimensional structural information available is confined to that of the unoccupied receptor. In this case, the binding site first needs to be identified. Of the many binding sites that have been found in proteins, most are situated in clefts or indentations in the protein surface, and these should be sought on the receptor as the most promising areas to be examined. An additional guide comes from knowledge of the effects of mutagenesis

giving indications of where residues affecting binding are located in the sequence and, hence, in the folded structure.

The reliance on having a three-dimensional structure for the binding site that has been determined by X-ray or other methods could become less important when methods for predicting the three-dimensional structure of proteins based simply on amino acid sequence become sufficiently advanced. It is beginning to look as though there might be only a limited repertoire of patterns of protein structure; it is perhaps pertinent that in hemoglobin, mutants are known involving practically all the residues, but the three-dimensional structure and function shows little variation. Predictions of structure can thus be modeled on comparison with an experimentally determined structure followed by refinement. This substantially reduces the number of degrees of freedom that need to be considered in the folding program. An example of this is the way the discovery of the transmembrane structure of bacteriorhodopsin was carried over to provide a realistic postulation of a similar set of seven transmembrane helices for adrenergic, muscarinic, and other membrane-located receptors. But note that so far, this has not led to reliable prediction of the three-dimensional structure of the extracellular and intracellular parts of these proteins. This is a disappointment because these are the very areas that constitute the binding sites for the substances we are interested in and that provide the basis for specificity. The need for finding how to fold these peptide loops is vital and, it is hoped, may be possible before long. The problem of the receptor structure complementary to an agonist is one that would be intermediate if the structure of the unoccupied receptor or the complex with an antagonist is known, because this becomes again an exercise, albeit a more complex one, in refinement of trial agonist-receptor complexes.

Another question is how to deal with the new regulatory substances being uncovered. Many of these are proteins or peptides, and therefore they have obvious bioavailability or kinetic limitations as drugs. A great deal of thought has gone into finding nonpeptide equivalents. Although some emphasis has been given to the more conservative approach of replacement of some of the amino acids with novel pseudo–amino acids or on combinations of amino acids with other frameworks, bolder spirits have been encouraged by the existence of morphine as an equivalent for the opioid peptides to treat these receptor sites as similar in principle to those for smaller regulators. Indeed, this has been thoroughly justified because, as a result of conventional screening methods, potent compounds have been found for angiotensin (Losartan and later compounds), oxytocin, gastrin, endothelin, and other peptide receptors among molecules no different in kind from those that would have been developed for small molecule receptors. One of the most obviously desirable peptide equivalents would be for insulin because so much is known about its structure and that of its receptor.

The approaches to new drug development I have outlined are being pursued vigorously and imaginatively in drug companies and in academic medicinal chemistry groups, and they are likely to produce many novel ligands. Despite their promise, it would not be expected that that they would have made any great

impact on the appearance of new drugs in therapy as yet, because the total drug development process is a long one and the discovery of new leads, and even the development of good derivatives, is only a small part; poor bioavailability, toxicity, and failures in clinical trials put paid to many hopefuls.

We live in a time when even large pharmaceutical companies feel insecure and amalgamate to become even larger, when they rely on only a few blockbusters to make their profit and have to live with a short period of patent protection. This means that even more than in the past, the targets they will concentrate on are those where a big impact can be made: cancer, AIDS, Alzheimer's, psychoactives, etc. It will be fascinating to see how the new approaches open up possibilities in these fields. Alas, there will be many interesting new compounds that will never merit economic development but would be fascinating experimental tools for the pharmacologist. It is to be hoped that they will not simply molder away in company archives.

LITERATURE CITED

1. Goldstein A. 1997. A rewarding research pathway. *Annu. Rev. Pharmacol. Toxicol.* 37:1–28

1a. Burgen ASV, Dickens F, Zatman LJ. 1949. The action of botulinum toxin on the neuromuscular junction. *J. Physiol.* 109:10–24

2. Burgen ASV, Terroux KG. 1953. On the negative inotropic effect in the cat's auricle. *J. Physiol.* 120:449–64
3. Burgen ASV. 1956. The secretion of non-electrolytes in the parotid saliva. *J. Cell Comp. Physiol.* 48:113–38
4. Martin K, Burgen ASV. 1962. Changes in the permeability of the salivary gland caused by sympathetic stimulation and by catecholamines. *J. Gen. Physiol.* 46:225–43
5. Burgen ASV, Kuffler SW. 1957. Two inhibitory fibres forming synapses with a single nerve cell in the lobster. *Nature* 180:1490–91
6. Enger PES, Burgen ASV. 1957. The effects of some anino acids on the perfused lobster heart. *Biol. Bull.* 113:345–46
7. Burgen ASV. 1966. The drug-receptor complex. *J. Pharm. Pharmacol.* 18:137–49
8. Taylor PW, King RW, Burgen ASV. 1970. Kinetics of complex formation between human carbonic anhydrase and aromatic sulphonamides. *Biochemistry* 9:2638–45
9. King RW, Burgen ASV. 1976. Kinetic aspects of structure-activity relations; the binding of sulphonamides by carbonic anhydrase. *Proc. R. Soc. London Ser. B* 193:107–25
10. Taylor PW, King RW, Burgen ASV. 1970. Inflence of pH on the kinetics of complex formation between sulphonamides and human carbonic anhydrase. *Biochemistry* 9:3894–903
11. Burgen ASV, Spero L. 1968. The action of acetylcholine and other drugs on the efflux of potassium and rubidium from smooth muscle of guinea-pig ileum. *Br. J. Pharmacol.* 34:99–115
12. Burgen ASV, Spero L. 1970. The effects of calcium and magnesium on the response of intestinal smooth muscle to drugs. *Br. J. Pharmacol.* 40:492–500
13. Hulme EC, Birdsall NJM, Burgen ASV,

Mehta P. 1978. The binding of antagonists to brain muscarinic receptors. *Mol. Pharmacol.* 14:737–50
14. Birdsall NJM, Burgen ASV, Hulme EC. 1978. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* 14:723–36
15. Birdsall NJM, Hulme EC, Burgen ASV. 1980. The character of muscarinic receptors in different areas of the brain. *Proc. R. Soc. London Ser. B* 207:1–12
16. Burgen ASV. 1986. The effects of ionic strength on cardiac muscarinic receptors. *Br. J. Pharmacol.* 88:451–55
17. Birdsall NJM, Berrie CP, Burgen ASV, Hulme EC. 1980. Modulation of the binding properties of muscarinic receptors: evidence for receptor-effector coupling. In *Receptors for Neurotransmitters and Peptide Hormones*, ed. G Pepeu, MJ Kuhar, SJ Enna, pp. 107–16. New York: Raven
18. Hammer R, Berrie R, Birdsall NJM, Burgen ASV, Hulme EC. 1980. Pirenzipine distinguishes between different subclasses of muscarinic receptors. *Nature* 283:90–92
19. Stockton JM, Birdsall NJM, Burgen ASV, Hulme EC. 1983. Modification of the binding properties of muscarinic receptors by gallamine. *Mol. Pharmacol.* 23:551–57
20. Burgen ASV, Jardetzky O, Metcalfe JC, Wade-Jardetzky N. 1967. Investigation of a hapten-antibody complex by nuclear magnetic resonance. *Proc. Natl. Acad. Sci. USA* 58:447–53
21. Feeney J, Roberts GCR, Brown JP, Burgen ASV, Gregory H. 1972. Conformational studies of some component peptides of pentagastrin. *J. Chem. Soc.* PII:601–4
22. Feeney J, Roberts GCR, Rockey JH, Burgen ASV. 1971. Conformational studies of oxytocin and lysine vasopressin in aqueous solution using high resolution NMR spectroscopy. *Nat. New Biol.* 232:108–10
23. Burgen ASV, Roberts GCR, Feeney J. 1975. Binding of flexible ligands to macromolecules. *Nature* 253:753–55
24. Metcalfe JC, Seeman P, Burgen ASV. 1968. The proton relaxation of benzyl alcohol in erythrocyte membranes. *Mol. Pharmacol.* 4:87–95
25. Birdsall B, Burgen ASV, Roberts GCR. 1980. Binding of coenzyme analogues to *Lactobacillus casei* dihydrofolate reductase: binary and ternary complexes. *Biochemistry* 19:3723–31
26. Birdsall B, Burgen ASV, Hyde EI, Roberts GCR, Feeney J. 1981. Negative cooperativity between folinic acid and coenzyme in their binding to *Lactobacillus casei* dihydrofolate reductase. *Biochemistry* 20:2186–95
27. Birdsall B, Burgen ASV, Rodrigues de Miranda J, Roberts GCR. 1978. Cooperativity in ligand binding to dihydofolate reductase. *Biochemistry* 17:2102–10
28. Gronenborn A, Birdsall B, Hyde E, Roberts G, Feeney J, Burgen A. 1981. 1H and 31P NMR characterization of two conformations of the trimethoprim-$NADP^+$-dihydrofolate reductase complex. *Mol. Pharmacol.* 20:145–53
29. Birdsall B, Gronenborn A, Clore GM, Roberts GCR, Feeney J, Burgen ASV. 1981. 13C NMR evidence for three slowly interconverting conformations of the dihydrofolate reductase-$NADP^+$-folate complex. *Biochem. Biophys. Res. Commun.* 101:1139–44
30. Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, et al. 1999. Pronounced pharmacologic deficits in M2 muscarinic receptor knockout mice. *Proc. Natl. Acad. Sci. USA* 96:1692–97
31. Rosoff ML, Wei J, Nathanson NM. 1996. Isolation and characterisation of the chicken m2 acetylcholine promoter region. Induction of gene transcription by leukaemia inhibitory factor and ciliary neurotrophic factor. *Proc. Natl. Acad. Sci. USA* 93:14889–94
32. Haddad E-B, Russell J. 1998. Regulation

of the expression and function of the M2 muscarinic receptor. *Trends Pharmacol. Sci.* 19:322–27
33. Weng G, Bhalla US, Iyengar R. 1999. Complexity in biological signalling systems. *Science* 284:92–96
34. Büscher R, Herrmann V, Insel PA. 1999. Human adrenoreceptor polymorphisms: evolving recognition of clinical importance. *Trends Pharmacol. Sci.* 20:94–99
35. Mahsood E. 1999. A consortium plans free SNP map of human genome. *Nature* 398:545–46
36. Luttrell LM, Ferguson SSG, Daaka Y, Miller WE, Maudsley S, et al. 1999. β-Arrestin-dependent formation of β2 adrenergic receptor-Src protein kinase complexes. *Science* 283:655–61
37. Ge M, Chen Z, Onishi HR, Kohler J, Silver LL, et al. 1999. Vancomycin derivatives that inhibit peptidoglycan biosynthesis without binding **d**-Ala-**d**-Ala. *Science* 284:507–11
38. Dooley CT, Chung NN, Wilkes BC, Schiller PW, Bidlack JM, et al. 1994. An all **d**-amino acid opioid peptide with central analgesic activity from a combinatorial library. *Science* 266:2019–22
39. Hünenberger PH, Helms V, Narayama N, Taylor SS, McCammon JA. 1999. Determinants of ligand binding to cAMP-dependent protein kinase. *Biochemistry* 38:2358–66
40. Koehler KF, Rao SN, Snyder JP. 1996. Modelling drug-receptor interactions. See Ref. 45, pp. 235–36
41. Itai A, Mizutani MY, Nishibata Y, Tomioka N. 1996. Computer-assisted new lead design. See Ref. 45, pp. 107–9
42. Birdsall B, Feeney J, Pascual C, Roberts GCK, Kompis I, et al. 1984. A 1H nmr study of the interactions and conformations of rationally designed brodimoprim analogues in complexes with *Lactobacillus casei* dihydrofolate reductase. *J. Med. Chem.* 23:1672–76
43. Giannis A, Rübsam. 1997. Peptidomimetics in drug design. *Adv. Drug Res.* 29:1–78
44. Cohen NC, ed. 1996. *Guidebook on Moleculer Modelling in Drug Design.* San Diego, CA: Academic