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Medicinal Chemistry Advances

Proceedings of the Seventh International Symposium on Medicinal Chemistry, Torremolinos, Spain 2 - 5 September 1980

Edited by

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Introduction

The VIIth International Symposium on Medicinal Chemistry was held in the Palace of Congresses of Torremolinos, Málaga, Spain, from the 2nd to the 5th september 1980, and was organized by the Sociedad Española de Quĭmica Terapéutica. The Symposium was attended by over 700 participants from 34 countries and consisted on 2 plenary lectures, 36 invited lectures on ten selected topics and 224 communications as posters.

In a moment like this of increasing difficulties for the research in Medicinal Chemistry we considered useful to have the views of qualified speakers about the present situation of drug design, discovery and development. Prof. A. Burger and Dr. F.P. Doyle talked in their plenary lectures about this subject from an academic and a pharmaceutical industry point of view, respectively.

The ten topics selected: Nucleosides in chemotherapy, Theoretical approaches to Medicinal Chemistry, Platelets and antithrombotic agents, Receptors, Antiviral agents, Antilipidemic agents, Respiratory system, Central nervous system, Enzyme inhibitors and Bioactive peptides, covered a wide field of interest within Medicinal Chemistry. In an attempt to bring together the different people involved in the discovery and development of drugs, the invited speakers were Medicinal Chemistry active chemists, pharmacologists, biochemists and physicians from both academic and industrial fields.

We want to express our gratitude to the members of the Organising Committee who chose the lectures of this Symposium and to the members of the Scientific Advisory Committee who helped them. Also to Mrs. Natividad Palacios de la Vega for her help in the preparation of this volume.

R. Madroñero
Chairman of the Organising Committee

F.G. De Las Heras and S. Vega,
Editors

Current Options in Drug Design

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ABSTRACT

The principal goals of medicinal chemistry are the discovery of "lead" compounds, their molecular modification, the preparation of drugs, and the explanation of their molecular mode of action. "Lead" compounds are discovered by screening but if a biochemical aberration can be established as an etiological factor in a given disease, metabolites involved in the biochemical lesion can become "leads". In a few cases, the reactions of a drug with active receptor sites, especially enzymic cofactors, have been analyzed and serve as guides for designing "lead" compounds. Molecular modification can be based rationally on bioisosterism. Some rules of bioisosterism were expanded and updated and have also received some aid from quantitative structure-activity studies.

KEYWORDS

Bioisosterism; irreversible inhibitors; "lead" compounds; medicinal chemistry; metabolic blockade; metabolite analogues; molecular modification; monoamine oxidase inhibitors; pharmacological guidelines to drug design; quantitative structure-activity relationships; receptors; screening; suicide enzyme inhibitors; transition state analogues.

INTRODUCTION

It is a festive occasion for me to participate in the opening of the 7th International Symposium on Medicinal Chemistry. Thirty-two years ago we started to schedule symposia on medicinal chemistry in the U.S.A., but even in 1951 patient persuasion was needed to be allowed to call the first edition of my book on medicinal chemistry by that title, since medicinal chemistry was still confused with analytical and diagnostic methods of chemical control in hospitals. Today we feel happy that our topic is approaching the scope of an exact science, with a record of proud achievements in the conception, development and understanding of hundreds of important drugs in almost all fields of therapy. If the literature of the last few years is a portent of the future we can be highly optimistic about the role medicinal chemistry will play in collaboration with other experimental therapeutic sciences and in the service of medicine.

Our role in this team effort is to create and furnish medicinal agents for biochemical and biological experimentation, and for therapeutic or prophylactic use in disease. This used to be done by trial and error or by serendipity. These

are wasteful methods but they have stood us in good stead in the past when augmented by intuition and experience in a specialized confine of chemical structures. It has been said in this regard that "ideas have to be fruitful, they do not have to be completely right" (Nathan, 1976).

As we have unraveled a few of the biochemical events in normal and abnormal physiology, it has become possible to limit screening programs to analogues, however structurally far-fetched, of natural metabolites. Educated guesses made by one medicinal scientist or a committee of several investigators are of some value in such selections. Indeed, one no longer wastes time on trying to solve therapeutic problems in areas of disease where no initial biochemical guidelines are available.

THE DISCOVERY OF "LEAD" COMPOUNDS

The most difficult problem of medicinal chemistry is the discovery of "lead" compounds. Only in very few cases have "lead" compounds been elaborated by deliberate drug design. One such example is the planning of pyridine-2-aldoxime methiodide (2-PAM) in nucleophilically reversing phosphate ester inhibition of acetylcholinesterase (Wilson, 1955). Another approach is the design of irreversible enzyme inhibitors whose alkylating groups might react with regions not directly involved in the active sites of an enzyme (Baker), 1967). A suggestion for partial de novo drug design is embodied in the discovery that individuals predisposed to lupus erythematosus are slow genetic acetylators. Pre-acetylation of various amines, or trial of non-acetylable compounds for anti-lupus activity should limit the randomness in screening experiments (Reidenberg, 1980).

In most other cases, random screening has been the source of "lead" compounds for further drug development. To be sure, these empirical searches have been limited reasonably in the case of natural products by giving preference to those with therapeutic folklore. In the case of synthetic substances, intuitive selection has also played a role, choosing those that have some structural resemblance to compounds of known biological activity. Quantitative structure-activity relationships may also be called in for help under special circumstances.

The time-honored search for "lead" compounds among natural products has experienced a renaissance during the last quarter of a century although the yield of therapeutically useful natural drugs has been low. Still, there is the lure that both plant or microbial metabolites, and mammalian metabolites that play a role in disease, have arisen from the same fundamental biochemicals which occur in the oxidative cycles and other basic reactions. Thus the chances that foreign natural products will have some relationship to disease-related vertebrate metabolites are better than for synthetic chemicals without biochemical analogy. In addition, the purely chemical interest in natural products, the outguessing of Nature's reasons for biosynthesizing them in botanical and microbial species, and the seductive voice of therapeutic folklore are incentives for working in this field.

An activity very similar to natural products research is the structural elucidation and quantitation of drug metabolites in animal and human blood, tissues and excreta. These studies not only shed light on metabolic activation and deactivation of drugs but have to fulfill legal requirements concerning drug metabolism before approval for the marketing of a drug is secured from regulatory agencies.

Lead compounds from screening programs will continue to turn up among many classes of compounds but this type of research is essentially chemically oriented and is not based on biological considerations. Even the screening of antibiotics from

molds and other microbes relies on therapeutic hopes rather than on biochemical reasoning.

A valid biochemical working hypothesis is provided by the metabolite analogue theory; it depends on the understanding of biosynthetic steps in enzymic reactions with a bearing on disease processes. The inhibition of a given enzyme in different cell species has to rely either on selectivity of drug distribution, or more hopefully on the presence of different isozymes in the respective cells. The most promising cases of differences exploitable in essential enzyme systems are found in the biochemistry of invasive cells such as parasitic, protozoal and bacterial organisms which have different isozymes for the same metabolic reactions. Such differences have been made use of in the inhibition of dihydrofolate reductase, of trypanosomal alpha-glycerophosphate dehydrogenase and a few other cases.

Another way to discover "lead" compounds is to observe secondary effects of a drug which may have a bearing on diseases not related to the drug's original purpose. The regulatory demands for the reporting of all observable effects have alerted experimental biologists to look out for any activities that might modify or detract from the primary utility of a drug. Examples include the antitrypanosomal action of the xanthine oxidase inhibitor, allopurinol, which is due to the toxic action of oxipurinol; the alpha-adrenergic agonist activity of the emetic alkaloidal product, apomorphine; and the antihyperglycemic and diuretic actions of antibacterial sulfanilamides. Such observations are essentially made by pharmacologists, and the medicinal chemist contributes only after the original discovery of a new pharmacological activity, emphasizing this activity by molecular modification.

When a drug is patterned on the structure or the function of a metabolite associated with a disease, the first few compounds designed on this basis have sometimes been regarded as "leads". In reality, they are molecular modifications of the damaging metabolite. For example, the reported development of histamine-2 receptor antagonists with its mix of physical-organic and medicinal chemistry (Brimblecombe, 1978) had modeled its original ideas on the imidazole structure as an archetype. In a series of simply substituted histamine derivatives it became apparent that H_2 receptor antagonism was reinforced where 1,3-prototropic shift, i.e. ring structure tautomerism, was favored. From physicochemical considerations and molecular models it could be concluded that 4-methylhistamine, with its small neutral electron-releasing group near the side chain position, produced the most favorable prototropic shift, and thus 4-methylimidazole became the "lead" for further modifications. That it is not a sine qua non necessity has since been shown by the potent activity of ring analogues such as furan derivatives which no longer offer the possibility of prototropic shift.

Compounds designed as structural analogues of natural metabolites are frequently competitive and reversible drugs. Their duration and effectiveness depend on the concentration of the natural substrate that they are supposed to displace. The classical way to overcome reversibility is to attach an alkylating group to the molecule of a drug so that a covalent bond to biomacromolecules can be established. Another idea is to synthesize structural analogues of transition states of a substrate. Additional current proposals concern enzymatic activation of inhibitors. Such compounds are equipped with a reactive, often unsaturated group, or a group convertible to an unsaturated group during reaction with an enzyme. This reactive group should be located so as to engage a function near, but not at the active site of the enzyme. Variations of this approach are affinity-labeling agents such as aryl azides with a masked group that can be activated photolytically at the active site.

Thus, while we have a few guidelines to operate more rationally than we did 50 years ago, an important link to macromolecular biochemical pharmacology is still missing. We can find chemicals with which to hit biochemical targets but we do not yet understand these targets adequately, be they active sites of enzymes or drug receptors or recognition sites for shapes and functions of drug molecules.

Three symposia at this meeting will deal with this question: theoretical approaches to medicinal chemistry, receptors, and enzyme inhibitors. I would like to mention briefly a few current approaches to such investigations.

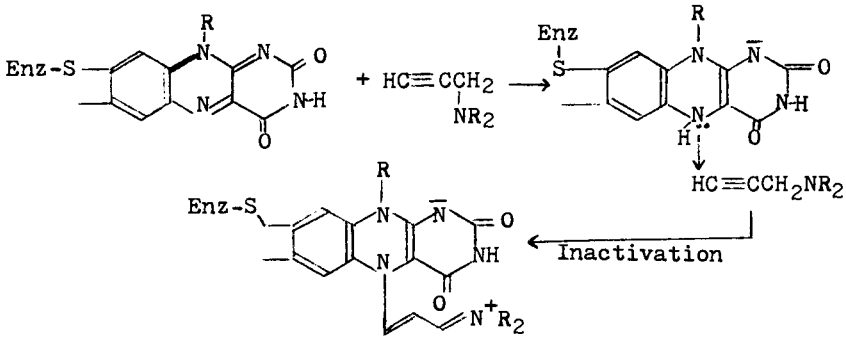
DRUGS AT RECEPTOR SITES

The recognition and quantitative estimation of receptor sites by means of autoradiography or fluorescence spectroscopy of radioactively or dyestuff-labeled drugs are now well-known procedures. One can also estimate the shape and the distances between ligating groups on the receptor by measuring the complementary steric and electronic distribution of the recognition features (pharmacophores) of several drugs which exert a similar biochemical or biological effect. For our purposes, a drug receptor shall be any biomolecular site at which a drug binds or is recognized in eliciting a biological response. It may be a polypeptide or lipoprotein on a membrane, an active site of an enzyme, or an intercalation groove or alkylating site of a nucleic acid (Gund, 1979). The traditional pharmacological studies of receptors do not, however, contribute to the understanding of receptor structure but only to that of receptor function. For example, beta-adrenergic receptors appear to be a subpopulation of norepinephrine receptors. The effect of antidepressant drugs on slowly decreasing the norepinephrine receptor-coupled adenylate cyclase may be a function of such agents and account for the slow clinical onset of their action (Sulser, 1979).

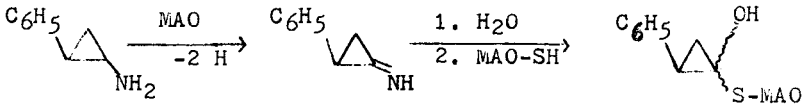
In a few cases, the three-dimensional structure of a drug receptor and its interaction with both substrates and inhibitors can be seen more clearly (Kuntz, 1980). For example, the placement of both dihydrofolate and methotrexate in a deep cavity of dihydrofolate reductase can be discerned in molecular models as well as from X-ray diffraction patterns of the respective complexes (Matthews, 1977). Computer-assisted memory can help to visualize the tumbling of the inhibitor to different positions that might presage molecular modification of subsequent candidate compounds.

Similarly, the inhibition of prostaglandin cyclooxygenase by indomethacin can be illustrated by fitting the inhibitor on the map of the active site of the enzyme (Gund, 1979).

Another case is that of the membrane-bound flavoprotein enzymes, monoamine oxidases A and B, which regulate the levels of biogenic amines and perhaps modify behavior; some MAO inhibitors are useful antidepressants. Of these, pargyline (a propargylamine), tranylcypromine (2-phenylcyclopropylamine) and some aralkylcyclopropylamines act as suicide enzyme inhibitors, that is, they react with the enzyme to give products that combine covalently, more or less irreversibly, with an active site or cofactor of the enzyme to render it ineffective. Several mechanisms by way of carbanions, free radicals and allenic intermediates have been proposed to explain the formation of the rigorously characterized adducts to the flavin moiety (Maycock, 1976).



By contrast, the suicide inhibition by tranylcypromine involves the addition of an intermediate, 2-phenylcyclopropanone or its imine, to one of the essential thiol groups of the subunits of MAO (Singer, 1979).



There are many other enzymes whose causative connections to diseases have been recognized. Among them are carbonic anhydrase which, depending on its anatomical distribution, regulates renal and ophthalmic processes; and adenylate cyclases which, contingent upon their sources in various organs, may rely on different hormonal factors and ions for activation. Inhibitors of these cyclases might be ionophores or chelating agents which remove the needed ions from the scene.

We usually postulate that drug receptors not only alter rates of biochemical reactions, as in the case of active sites of enzymes, but lead to observable biological changes. An overlapping of such requirements with purely enzymic properties is seen in plasminogen activator (PA), a serum protease which cleaves arginine peptide bonds and converts the inactive zymogen, plasminogen, to plasmin. Plasmin itself is a proteolytic enzyme associated with tissue breakdown, ovulation, implantation of trophoblasts in the uterus, mammary gland involution, metastases and other neoplastic phenomena (Rohrlich, 1979). Other enzymes that activate plasminogen are urokinase and streptokinase. PA is inhibited by substances of diverse structure, and this suggests that specific medicinal agents for some of these conditions may be found among PA inhibitors.

Such observations touch upon a fundamental question of drug action: how can Nature or Evolution anticipate that some medicinal chemist will devise a new chemical tomorrow, that this compound will have therapeutic activity and become a usable drug? We must realize that many nutrients and endogenous metabolites can be toxic at high concentrations, and that many environmental impulses may be noxious to living organisms. The 20-odd neurohormones already found, and others still hidden or not enough stabilized on chromatographic columns, may provide partial answers to this question of drug action. Some of these hormones which elevate the pain threshold will be the subject of one of our symposia. Molecular modification of the endorphins has already resulted in synthetic analogues more potent and more specific than the natural hormones (Miller, 1979). We should perhaps turn to constituents of our diet in a search for "leads" for selectively toxic pharmacological agents.

MOLECULAR MODIFICATION

Nature practices molecular modification in many biosynthetic processes. In the plant kingdom, related alkaloids occur in most alkaloid-bearing species. Molds frequently metabolize nutrients to different derivatives of the same antibiotic moiety. Structurally related hormones are the rule among steroids, thyroid hormones, endorphins, adrenergic amines and many others. Regularities in the variations of biological activity found in these compounds can be traced directly to differences in the chemical structure of the substances. Similarly, isozymes which may be regarded as molecular modifications of one another react differently to chemical inhibitors.

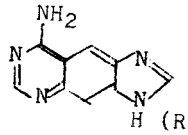
In medicinal chemistry, molecular modification is over 100 years old. It was initiated by Crum-Brown and Fraser (1868-1869) in their studies on the behavior of quaternary ammonium ions at the neuromuscular junction. It is interesting that these early observations on structure-activity relationships occurred with synthetic compounds when the structures of the natural curarizing alkaloids were not yet known. This paved the way for the study of other synthetics such as the analgetic-antipyretic and antiinflammatory aminopyrazolones (Knorr, 1883) and the hypnotic-sedative cyclic ureides (Conrad, 1882; Fischer, 1903). In these series, systematic structural modification was dictated primarily by synthetic expediency and reliance on chemical homology.

The first hopes that side effects of "lead" compounds could be decreased by molecular modification were raised among derivatives of salicylic acid, especially its peripherally acting esters such as phenyl salicylate (salol) (Nencki, 1886) and aspirin (Dreser, 1899). The durability of these ideas in drug design is attested by the continuing interest in salicylate esters as prodrugs (Paris, 1979), almost 90 years later. The lapse of 70 years before a biochemical explanation for the action of salicylates was proposed (Vane, 1971; Ferreira, 1974) may give us pause but also raise the hope that the greatly advanced state of biochemistry will not let delays like that happen again.

The same year that aspirin was introduced as an obviously less polar derivative of salicylic acid, Meyer (1899) and Overton (1901) studied drugs which physically disorganize the cellular environment. The depressant activities of such drugs and their partition coefficients between water and water-insoluble solvents (1-octanol was chosen 60 years later) (Hansch, 1964) are directly related. This layed the groundwork for all subsequent experiences in physical-organic medicinal chemistry and shaped quantitative structure-activity relationships. In attending the symposium on QSAR tomorrow we should remember that the origins of QSAR go back to the turn of the century.

The feeling that increased lipophilicity facilitates the crossing of membranes by drugs has deeply affected drug design (Kubinyi, 1979). Hydrophilic structures can be equipped with aliphatic or aromatic moieties on the "back side" of the molecule where such groups will not interfere with active-site-directed functions.

If the prototype structure, especially of a metabolite agonist, contains flexible areas, computer memory may average out many possible conformations by twisting about a single bond and estimating the energy content of the more likely shapes. This increases in complexity and loss of accuracy as the number of atoms and flexible bonds rises. Therefore intuitive interpretations of spacial requirements have not lost their value. An example of successful intuition is the stretching of adenine (or its ribosides) by inserting an additional ring between the pyrimidine and imidazole systems, for instance, a benzene ring.



Since adenine is needed in a large number of enzymic reactions as AMP, cAMP, ADP, ATP, NADP, etc., a study of its specificity for catalytically active sites is of great interest. The aromatic "homologue" shown can indeed replace adenine in many enzymic reactions; this has led to a mapping of permissible distances between sites requiring structural features of adenine (Leonard, 1975).

In contrast to the general goal of molecular modification, i. e. improvement of potency and spectrum of activity, prodrugs represent modification with retention of intrinsic activity (Fung, 1979). They usually contain blocking moieties at functional groups which change the lipophilicity and permit reconversion to the parent drug at controlled rates. This affects the pharmacokinetics of the agent, especially absorption, distribution, renal clearance, and other rates of elimination.

In many cases of "anti" drugs, the structure of such agents is patterned on that of the metabolite which the drug is to block. A different purpose of blocking groups is the prevention of premature degradation. Many compounds are epoxidized and hence hydroxylated in their bioconversion, sometimes so quickly that their activity at drug receptors cannot manifest itself. Blockade of molecular positions susceptible to such oxidations prolongs activity or even lets activity come to the fore. But such blocked compounds are new drugs with unknown toxicities and must be studied from a fresh start.

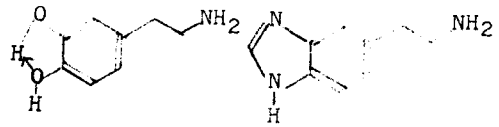
On successful example of metabolic blockade in drug design is the development of the antimalarial agent, mefloquine, which is curative in chloroquine-resistant infections by Plasmodium falciparum (Sweeney, 1979). The vinylquinuclidine section of quinine had been simplified 42 years ago (Ainley, 1938) but the resulting piperidyl alcohols suffered from the same metabolic deactivation as the parent alkaloid, i.e. oxygenation at position 2'. Blocking of this position by aromatic moieties inevitably gave compounds which photosensitized the test animals. Replacement of aromatic groups by the electron-withdrawing CF_3 group considerably reduced this phototoxicity (Pinder, 1968). Following this "lead", Ohnmacht, Patel and Lutz (1971) introduced additional trifluoromethyl groups into other positions of the quinoline ring until, in the 2', 8'-bistrifluoromethyl derivative, a virtually non-photosensitizing but effective drug, mefloquine, was found.

The combination of chemical similarity, however tenuous, and similar electronic, steric and polar expressions of similarly-acting compounds is known as bioisosterism (Friedman, 1951; Burger, 1970). This concept has superseded earlier and more restrictive definitions of isosterism (Langmuir, 1919; Grimm, 1925; Erlenmeyer, 1932). Hansch (1974) tried to redefine bioisosteres as compounds which elicit identical biochemical or pharmacological responses in a standard test system regardless of their chemical structures, as long as they have certain physical properties in common, and "because organic formulas do not express reaction rates". This would imply that reactivity would override structural and steric considerations, whereas too many examples are known where steric features are more important in structure-activity relationships than other factors. Hansch also adds that a standard test system may be "an enzyme, mouse or man" although this does not take into account biological inactivity of a compound which never reaches a receptor in adequate concentrations because of side-tracking at metabolic and other sites of loss. Such a definition would, however, cover drugs assayed for an in vivo effect without regard to differences in their mechanism of action.

No doubt, complete likeness of several significant physical properties should be the hallmark of molecules with like biological activities. Beyond that, the practical purpose of bioisosteric replacements is not necessarily to give us like-acting compounds but substances with modified and hopefully improved biological properties. Suppose we could identify biological similarity from measurements of several physical properties; a catalogue of hundreds of thousands of compounds listed according to their hydrophobicity, polarity and other pertinent physical data would have to be consulted for meaningful comparisons, and such an extensive catalogue is not likely to be available in our time. But suppose we could identify iproniazid and pargyline as closely similarly acting MAO inhibitors based on physical properties. What reasoning would lead us to tranylcypromine which, for the sake of discussion, had not yet been discovered? We need other support in drug design, not only computer-stored physical data. Even screening of large numbers of random test compounds in a battery of in vitro enzymes gives only partial information that could be carried over to in vivo conditions. For every of the few successes in quantitative structure-activity relationships, many more disappointments in predictive drug design have been registered. The pharmaceutical industry especially would like nothing better than to be able to rely on data from one science to be predictive without having to combine chemistry with biology, or with macromolecular biochemistry in which sufficient structural knowledge is not yet at hand.

Among the successful newer applications of bioisosterism are functional replacements of phenolic hydroxyls by other groups of similar pk_a. Among them are CH_2OH, CH_2SO_2R, and $NHSO_2CH_3$. They share ionizable protons on the atom attached to the aromatic ring as it is also seen in phenolic OH. Apparently their increased bulk is still acceptable as long as similarity of dissociation is preserved. In an analogous case, carboxyl has been replaced by tetrazolyl in several instances with retention of biological activities.

Ring structures have been exchanged for non-cyclic ones in interesting cases. The flat hydrogen-bonded catechol ring (Pauling, 1936) of dopamine can be imitated by a benzimidazole structure with fair retention of dopamine agonist activity (Arnett, 1978).



Similarly, the hydrogen-bonded non-nitrogenous quasi-ring of cycloheximide is equivalent to the covalently bonded basic isoquinoline ring of emetine. The two drugs, and especially 4-hydroxy- and 4-acetoxycycloheximide, exhibit virtually identical biochemical effects on the inhibition of protein assembly at the ribosomal level (Grollman, 1966).

Some other ways of conducting molecular modification will be discussed at this symposium. Those that have been described well in the literature are now standard procedure. An indication of how firmly embedded these concepts have become is provided by the November 1979 issue of the Journal of Medicinal Chemistry. Of the 30 papers in that issue, ten made use and give references to bioisosteric principles. But only in a few lucky cases has the first compound designed by planned molecular modification become a useful pharmacological agent; in almost every instance, additional empirical modification is necessary to achieve acceptance in medicine.

Thus, two main activities, skillful performance in bio-organic chemistry and the rationalization of drug design, have established medicinal chemists as independent scientists. Because pharmacological studies are precursors of clinical pharmacology, experimental biologists live in the shadow of increasing governmental regulation of drug-related research and development. Medicinal chemists have an advantage in this regard. Their task is to be scientists without an immediate and direct impact on clinical application. We should be grateful for this opportunity to do work of constantly varying interest, to be part of the exploration of basic biochemistry to which we furnish revealing agonists and antagonists, and to feel that ours is the rewarding excitement of being at the unfolding edge of new discovery with beneficial and therapeutic overtones that are applied to a happier life of mankind.

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Medicinal Chemistry and Current Problems in Drug Development

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ABSTRACT

The revolution in the treatment of disease that has taken place over the last 50 years is reviewed, with particular emphasis on the improved treatment of bacterial infection through the development of a wide range of beta-lactam antibiotics. Recent advances in this field are exemplified. The essential contribution of clinical pharmacology studies in the uncovering of novel drug activities is related to the difficulty of predicting such activities from laboratory studies. Some of the problems associated with escalating costs and time periods for bringing new drugs from laboratory to patient are outlined. Future prospects are briefly reviewed.

KEY WORDS

Therapeutic revolution; beta-lactam antibiotics; drug discovery; clinical pharmacology; drug development time-scale.

INTRODUCTION

The revolution in the treatment of disease

I have no doubt that historians would trace the beginning of disease treatment back to the Egyptian god, Asklepius Imhotep, who was supposed to have lived in the year 2780 BC, and who turned up again some 2400 years later (400 BC) as the Greek god, Asclepius. This gentleman, who was reputed to have studied medicine under Cheiron, a centaur, travelled about Greece healing the sick with prescriptions prepared from herbs and plants. In his temple at Epidaurus, which is conveniently sited next to the famous theatre, are recorded recoveries from such diseases as tapeworm and sterility. However, wherever one chooses to place the origins of disease therapy, I am sure that most medicinal chemists will agree that the real revolution in such therapy has arisen in this century, and is not much more than 50 years old.

It is significant that this therapeutic revolution has occurred simultaneously with a revolution in transport, through development of efficient internal combustion and jet engines, the revolution in mass-communication technology through radio and television, and the revolution in the development of computers and micro-electronics.

Scientists of today can move around the world faster, reach their scientific conclusions much faster, and be reported by the mass-media faster, than their colleagues of 50 years ago.

If we look back for a moment at the medicines that were available to the doctor at the turn of the century (Fig. 1) and up to the early 1930's (Fig. 2) we can see what few drugs he had at his disposal.

Drugs available before the year 1900	
Iodides	- goitre
Bromides	- sedatives
Nitrites, Digitalis	- heart disease
Iodoform, Phenol	- antiseptics
Mercuric chloride	- anthrax
Cocaine	- local anaesthetic
Caffeine	- diuretic
Methylene Blue, Quinine	- malaria
Aspirin	- antipyretic
Atropine	- mydriatic
Morphine, Codeine	- analgesic

Fig. 1

Drugs available 1900-1935
1902 Theophyllin - diuretic
1903 Veronal - first barbiturate hypnotic
1907 Trypan red - trypanosomiasis (animals)
1909 Trypan blue - babesia
1912 Phenobarbital - epilepsy
1913 Emetine - amoebic dysentery
1916 Suramin - human trypanosomiasis
1917 Hypochlorites - antiseptics
1922 Purified insulin - diabetes
1926 Pamaquine - antimalarial
1927/28 Vitamin C isolated
1933 Vitamin C synthesised

Fig. 2

The British Pharmacopoeia of 1932 in fact contained just 36 synthetic drugs including aspirin, phenacetin, and phenobarbitone, all of which had been available as synthetic organic chemicals for many years prior to their use in medicine. The British Pharmacopoeia today contains some 600 such drugs, the majority of which have been introduced within the last 20 years. The 1976 Merck Index (the medicinal chemist's bible) has 9,856 entries of individual drugs, and lists nearly 800 individual Pharmaceutical Companies most of which have active R & D groups.

Perhaps the most striking examples of the changes that have taken place in disease therapy are shown in Figs. 3 and 4, which refer to changes in mortality to various diseases brought about by the use of vaccines and antibiotics. There are, of course, many other examples of such changes relating to heart disease, mental disease, diabetes, etc. which although not as dramatic as the effect on infant mortality, do show up as a reduced need for hospitalisation in the case of some diseases (Fig. 5) and increasing life expectancy (Fig. 6) in both developed and developing societies.

The contrast between the period of the mid-1930's and today was summarised most eloquently by Sir John (now Lord) Richardson at a private conference held by my Company some 10 years ago. Lord Richardson said, and I quote:

> "I so well remember standing at the end of one of those long Nightingale-type wards at St. Thomas's Hospital (London) and looking back at the quiet, good order of it, and at the bright colour from the flowers and the young nurses, and saying to the Sister with despair in my heart ... we know a lot about each of those people but there isn't one for whom we can do anything really effective. It was 1937, and a moment of great disillusion - disillusion about my newly acquired knowledge

"and the confidence in my powers that it had engendered. How different it is now. If, at the end of a long round, there are two or three patients for whom I feel that nothing can be done, my depression and irritability is great but is no longer because there is nothing that we can do for most patients but because there is some sort of limitation to it."

Such expressive words as these summarise for all of us the driving force that makes the frustrations of day-to-day research in medicinal chemistry so worthwhile and is surely the basic reason why we continue to try to solve the problems that confront us today.

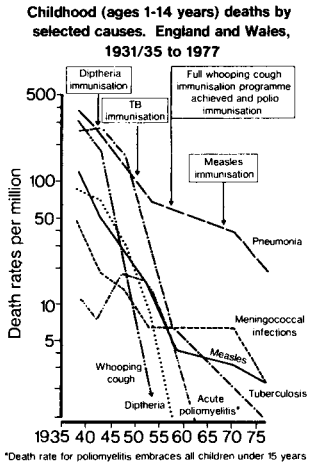


Fig. 3

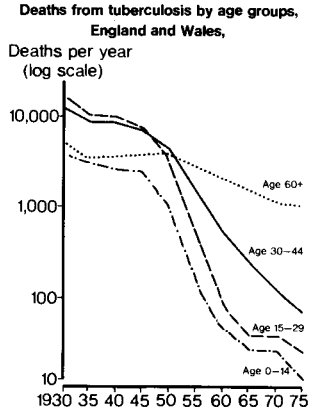
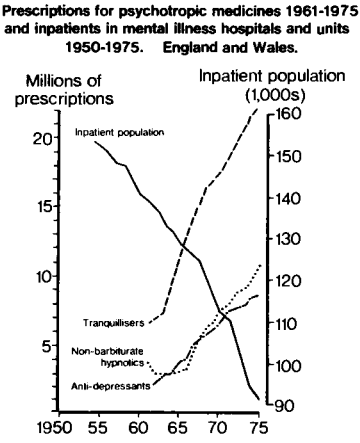


Fig. 4



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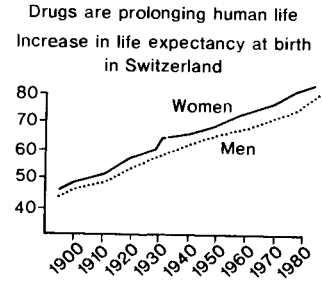


Fig. 6

The chemist as a manipulator of molecules

Throughout all these changes there seems to me to be one recurring theme, and that is, the facility with which the organic chemist, often with the most tenuous of leads, is able - to quote words used by the late Sir Alexander Fleming in a B.B.C. radio broadcast in 1943 - "to fasten on the molecule and modify it so that derivatives will appear more powerful or with wider application and diseases now untouched will be conquered". He was in fact referring to the penicillin molecule and it is appropriate that I should use examples from this field to demonstrate the versatility that the medicinal chemist has been able over the last 20 years to build into the basic beta-lactam structure.

As you all know, the discovery of Penicillin G, by Chain, Florey, and Fleming, initiated a whole era of antibiotics derived from mould metabolites. This first discovery of Penicillins G and V led to later discoveries of chloramphenicol, the macrolides, the aminoglycosides, the tetracyclines, the polypeptides, and many other antibiotic substances. In much of this earlier work the medicinal chemist was involved mainly in structural elucidation and attempted synthesis but did not have available the basic molecules for much molecular manipulation.

The first real opportunity for this came with the discovery in 1957 of 6-aminopenicillanic acid by my colleagues and I in the Beecham laboratories at Brockham Park, which was followed by the parallel work on 7-aminocephalosporanic acid by Florey, Abraham, Newton, and their co-workers at Oxford, at the Glaxo laboratories, U.K., at Eli Lilly laboratories, U.S.A., and in several other centres. The variations achieved during the course of both these developments are shown in Figs. 7 - 12.

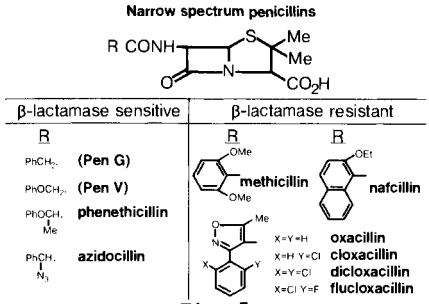


Fig. 7

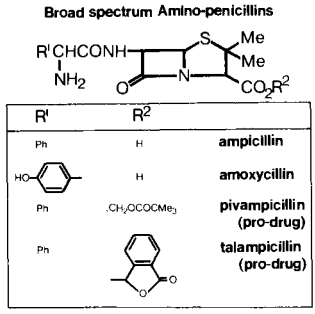


Fig. 8

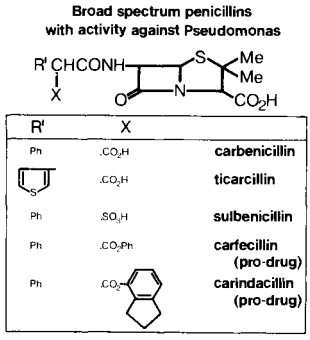


Fig. 9

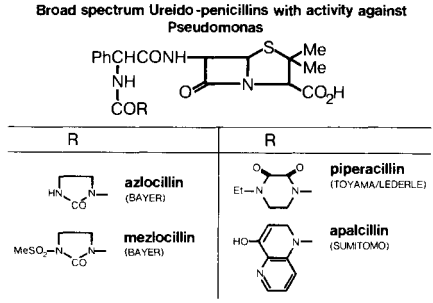


Fig. 10

Cephalosporins - Broad Spectrum (Parenteral)

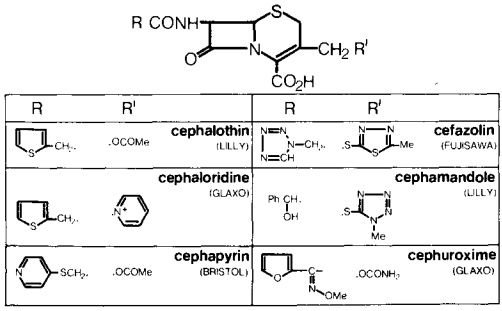


Fig. 11

Cephalosporins Broad spectrum (oral)

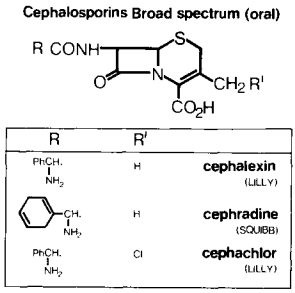
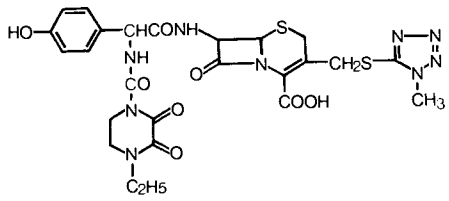


Fig. 12

The increasing complexity of the side-chains of derivatives of 6-APA and 7-ACA, together with the variations in the grouping in the 3-position of the cephalosporins, probably reached their peak in the structure of cefoperazone (Toyama, 1551) shown in Fig. 13.



Cefoperazone T-1551

Fig. 13

In the early 1970's one would have been tempted to think that the chemistry of the beta-lactam ring system had been fully exploited by the discovery and commercial introduction of some 22 semi-synthetic penicillins and some 10 or so cephalosporins. However, while the chemists had been developing their many variations on the original themes, the microbiologists had also been hard at work looking for yet more sources of antibiotic inspiration.

The first new departure came from the Eli Lilly and Merck groups with the discovery of the cephamycins which led, in 1975, to the Merck semi-synthetic product, Cefoxitin (Fig. 14). Here we have a repeat of the cephalosporin story except that the organism (streptomyces clavuligerus) had introduced a 7-MeO group into the beta-lactam nucleus to form the basis for a series of 7-MeO cephalosporins. This was in fact the first indication of useful antibacterial beta-lactam structures arising from a streptomyces series, but it was rapidly followed by several further entries to the field.

In our laboratories at Brockham Park, in 1970, using specifically designed test systems, we discovered that two streptomyces species, streptomyces olivaceous and streptomyces clavuligerus, produced metabolites that would inhibit many beta-lactamases, the penicillin-splitting enzyme present in many forms of resistant bacteria. At about the same time, Merck workers discovered high antibacterial activity in another species of streptomyces, streptomyces cattleya, and work carried out by the Sanraku Company in Japan using streptomyces cremeus also

revealed a substance (PS5, Fig. 21) with similar broad-spectrum antibacterial properties.

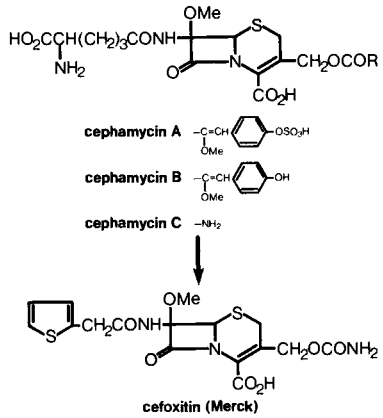


Fig. 14

The beta-lactamase-inhibiting substance from streptomyces clavuligerus was found by the Beecham workers to be a simple fused oxacyclic beta-lactam ring system (given the trivial name, clavulanic acid) which had the properties of inhibiting many beta-lactamases at low MIC values (Figs. 15 and 16). Formulations of clavulanic acid with a number of biosynthetic and semisynthetic penicillins are under development in our laboratories in the U.K. and the first of these, a formulation of clavulanic acid with amoxycillin, will be marketed in several European countries under the trade name, Augmentin, during the course of the next 12 months. Some simple modified penam nuclei (Fig. 17) have also been shown to possess beta-lactamase inhibitory properties similar to that of clavulanic acid.

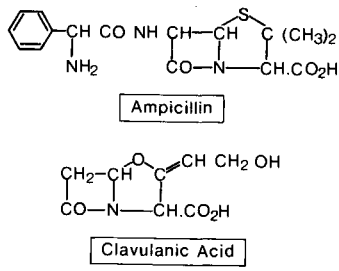


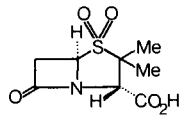
Fig. 15

Effect of clavulanic acid (C.A.) on the activity of amoxycillin against β-lactamase-producing organisms

Organism	Amoxycillin + C.A. (μg/ml)			C.A. alone
	0	1.0	5.0	
B.fragilis (28)	33	0.48	0.14	13.1
E.coli (100) *	5000	94.5	13.2	24.8
H.influenzae (15)	150	0.72	0.44	36.8
K.aerogenes (45)	315	1.75	0.89	33.2
K.aerogenes (32) *	5000	126	20	33.6
N.gonorrhoeae (6) *	40	0.18	-	5.6
Proteus sp (23)	433	11.6	4.2	62.9
Staph. aureus (35)	106	0.72	0.17	17.1

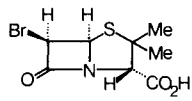
Geometric mean M.I.C. μg/ml
Numbers in parenthese indicate number of strains tested
* Plasmid-carrying strains

Fig. 16



CP-45,899
Penicillanic acid sulphone

English, A.R. et al., 1978, Antimicrob. Ag. Chemother., 14, 414.



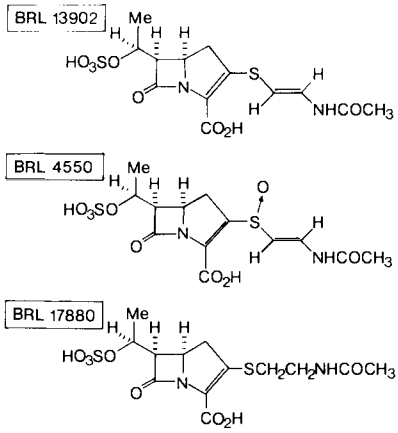
β-Bromopenicillanic acid

Pratt, R.F. and Loosemore, M.J., 1978 Proc. Nat. Acad. Sci., U.S.A., 75, 4145.

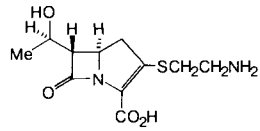
Fig. 17

The work with the other streptomyces species again indicated the existence of several beta-lactam structures associated with a carbocyclic beta-lactam ring system (the carbapenem ring) which, together with some of their antibacterial activities, are shown in Figs. 18 - 21.

STREPTOMYCES OLIVACEUS ATCC 21379



STREPTOMYCES CATTLEYA NRRL 8057



Thienamycin

Fig. 18

Antibacterial activity

Organism	MIC µg/ml		
	MM 4550	MM 13902	MM 17880
B. subtilis	3·1	0·1	0·2
Bacteroides		0·4	
Citrobact. freundii*	12	0·8	0·8
Ent. cloacae*	200	25	25
E. coli	6·2	0·4	0·4
E. coli*	15	1·6	0·8
Haemophilus	2·0	0·07	2·0
Kleb. aerogenes*	12	0·2	0·4
Neisseria	2·5	0·8	0·4
Pr. mirabilis*	3·1	0·2	0·4
Pr. vulgaris*	3·1	0·2	0·8
Ps. aeroginosa*	500	62	125
Sal. typhimurium	15	0·2	0·4
Serr. marcescens*	6	0·4	0·8
Staph. aureus	25	0·4	0·8
Staph. aureus*	50	1·6	1·6
Strep. pyogenes	3·1	0·05	0·05

* β-lactamase producing strains

Fig. 19

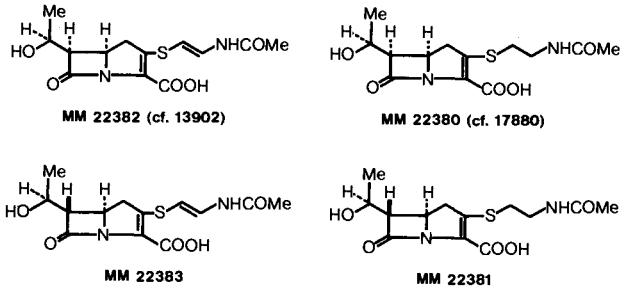


Fig. 20

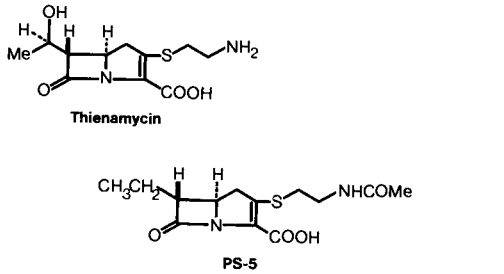


Fig. 21

It is not possible for me in this review to go into the many variations and somewhat complex stereochemical/structure activity relationships associated with these new ring systems, some of which have beta-lactamase inhibitory activity and some high antibacterial activity. There is no doubt, however, that the carbapenem nucleus, now that it has been shown to exist naturally, is already attracting the attention of the organic chemist and modifications of the parent compounds are being explored and synthetic routes to such structures are under investigation. As an example, at a recent scientific meeting in the U.S.A., Merck announced the total synthesis of their preferred derivative of thienamycin (MK 0787) with all the asymmetric carbon atoms in their correct configuration as in the natural product (Fig. 22). The chemists are also striking back in other ways. The Hoechst-Roussel group have synthesised and have just marketed in Europe a 7-ACA derivative (cefotaxime, Fig. 23) with high antibacterial activity (by injection) which represents a significant increase in antibacterial activity over earlier 6-APA and 7-ACA derivatives against some gram-ve organisms.

Perhaps, for the organic chemist, the peak of achievement in this field has been reached with the total synthesis by Shionogi of the oxacephalosporin (moxalactam, 6059-S) which is now undergoing clinical trial in cooperation with

the Lilly group (Fig. 23). 6059-S may be the last word in the best of all beta-lactam worlds since it contains the pOH phenyl group of amoxycillin, the alpha-CO_2H group of carbenicillin, the 7-MeO group of cefoxitin, the cyclic oxygen of clavulanic acid, and the 3-thiotetrazolo grouping of cephamandole.

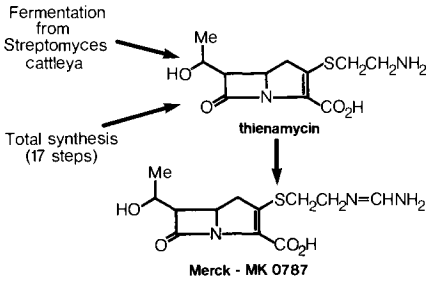


Fig. 22

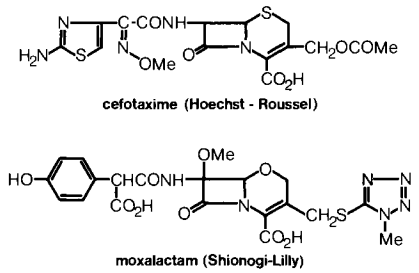


Fig. 23

To summarise the first part of this review, the ingenuity of the chemist and microbiologist over the last 20 or so years has provided a wide range of beta-lactam antibiotics containing 6 basic nuclei (Fig. 24) and an increasing width of spectra and intrinsic activity (Figs. 25 and 26).

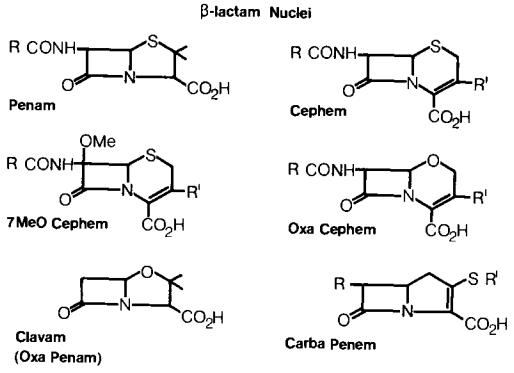


Fig. 24

Comparative activity of β-lactam antibiotics Typical MIC (μg/ml)

Organism	Penicillin G	Methicillin	Amoxycillin	Ticarcillin	Amoxycillin/ Clavulanic Acid
Staph. (sensitive)	0·02	2·5	0·05	0·5	0·1
Staph. (resistant)	R	2·5	125	25	2·5
E. coli (sensitive)	25	R	5	2·5	5
E. coli (resistant)	R	R	R	R	12·5
Klebsiella	250	R	125	250	2·5
Pseudomonas	R	R	R	25	250
Serratia	R	R	125	12·5	125
Proteus Indole+ve	R	R	R	5·0	5-250
Proteus mirabilis	5	R	1·25	1·25	1·25
Bacteroides	25	R	25	25	1·25
Strep. pyogenes	0·01	0·5	0·01	0·25	0·01
Strep. faecalis	2·5	25	0·5	50	1·25
Haemophilus	0·5	2·5	0·25	0·5	0·25

R = resistant - greater than 500μg/ml

Fig. 25

Comparative activity of β-lactam antibiotics Typical MIC (μg/ml)

Organism	Cephalothin	Cefuroxime	Cefoxitin	Cefotaxime	Moxalactam (6059-S)	Thienamycin	Olivanic acid (BRL 13902)
Staph. (sensitive)	0·2	0·2	1·25	1·5	10	0·02	1·5
Staph. (resistant)	0·2	0·5	2·5	1·5	50	0·02	1·5
E. coli (sensitive)	5	2·5	2·5	0·05	0·5	0·2	0·2
E. coli (resistant)	50	5	2·5	0·2	0·5	0·5	1·5
Klebsiella	5	2·5	2·5	0·02	0·5	0·5	0·5
Pseudomonas	R	R	R	25	12·5	5	50
Serratia	50	50	5-25	1	1	5	5
Proteus Indole+ve	500	25	5·0	0·1	1·0	5	0·2
Proteus mirabilis	5	0·5	2·5	0·02	0·5	5	0·2
Bacteroides	>100	>100	5-10	10	5	0·5	0·5
Strep. pyogenes	0·1	0·1	1·25	0·02	2·5	0·02	0·05
Strep. faecalis	50	250	250	250	250	1·5	5
Haemophilus	10	0·5	25	0·02	0·2	0·1	0·1

R = resistant - greater than 500μg/ml

Fig. 26

Serendipity and the clinician

I would not like to give you the impression, however much you would all like to believe it, that all new drug developments are made logically as the result of chemists, pharmacologists, microbiologists, all beavering away at structure/activity relationships and designing on a rational scientific basis new drug molecules with increasing activity, improved bioavailability, and lack of toxicity.

I know, and you all know, that this is only partially true and it will be many years before automated chemical synthesis, followed by automated bioassay, followed by computerised structure/activity calculations with a feed-back loop to the chemical synthesiser, will replace the synthetic ingenuity of the chemists, the skill and acute observation of the biologists including of course the clinical pharmacologists and the clinicians, together with the serendipity (or luck, if you prefer) that appears to exercise a benign influence over all. There are countless examples of such influence, ranging from the almost accidental synthesis of the

benzodiazepinone tranquillisers by Sternbach, in the Roche laboratories, the leap in the dark that led to the isolation of 6-APA in our own laboratories, and the confidence and personal courage that led Dr. Altounyan, of Fison, to test over 600 novel substances on induced bronchial asthma in himself in the search that resulted in the discovery of the anti-asthma drug. Intal.

Many important discoveries leading to significant advances in the treatment of disease in the past in fact have taken place in clinical laboratories, and I believe will continue to do so. The oft-quoted example of this is, of course, the story of the sulphonamide antibacterials which, through astute clinical observation, led to the sulphonyl ureas used in the treatment of diabetes and the sulphonamide diuretics used in the treatment of hypertension (Fig. 27). The way that research into one area of therapy frequently leads into discoveries in another, even although the original ideas may be of the "me too" variety, is something that the critics of molecular manipulation (or molecular roulette, as it has been cynically termed) still do not appear to comprehend.

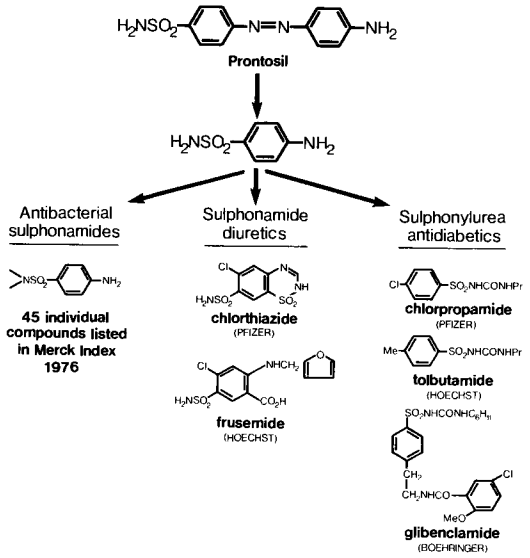


Fig. 27

The role of the clinical pharmacologist in drug discovery was re-emphasised recently by Professor Alistair Breckenridge, at the conference organised by the Society for Drug Research in March 1980 on "Risk Benefit Analysis In Drug Research". He drew attention to the essential input of the clinical pharmacologist when he pointed out how many important drug developments have stemmed from clinical observations of "side-effects" which have led to important new uses for drugs (Fig. 28).

Pharmacologists will no doubt argue that today it should be possible to detect such alternative activities of new substances from the pharmacological screening programme. However, there is still a very large gap between the laboratory animal and man, since absorption and excretion patterns (serum half-life) are often very different, metabolic pathways (detoxification) frequently differ quantitatively and qualitatively (Fig. 29), we still lack good animal models for many disease states (e.g. migraine, atherosclerosis, rheumatoid arthritis, allergic conditions).

Drug	Designed use	Discovered use
Propranolol	Angina	Hypertension
Sulphinpyrazone	Uricosuria (Gout)	Ischaemic heart disease
Phenobarbitone	Hypnotic	Epilepsy
Chlorpromazine	Antihistamine	Schizophrenia
Oestrogen/ Progestogen	Replacement therapy	Antifertility
Imipramine	Tranquilliser	Antidepressant
Probenicid	Penicillin blood level extender	Uricosuria (Gout)
Lignocaine	Local anaesthetic	Arrhythmia
Clonidine	Nasal vasoconstrictor	Hypertension (Migraine)

Fig. 28

Metabolic half-life (hrs) of various drugs in different species

Drug	Mouse	Rat	Dog	Rhesus Monkey	Human
Hexobarbital	0·3	2·3	4·3	-	6
Meperidine (Demerol)	-	-	0·9	1·2	5·5
Phenylbutazone (Butazolidin)	-	6	6	8	72
Antipyrine	-	-	1·7	1·8	12
Digoxin	-	9	27	-	44
Digitoxin	-	18	14	-	216

Fig. 29

Problems of drug development today

The inter-relationship that I have been attempting to highlight in this review, between the synthesis and biological testing of compounds in laboratory studies, the relationship of their activity in animals to their likely activity in man, is all part of the drug discovery process, with which you are all familiar. To this process must also be added the therapeutic ratio in relation to the disease being treated, and the cost of treatment to the patient or to the Health Services. No one part of this overall drug development process can operate in isolation (Fig. 30).

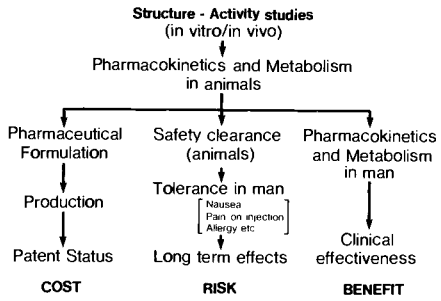


Fig. 30

During the past 20 years, the time period for drug development from laboratory bench to patient has become longer and longer, and more and more costly. This is a matter of very great concern to all medicinal chemists at the present time, and I would like to devote the final part of my review to the effect it is having on our research endeavours.

Concern over the safety of drugs is not a new phenomenon but came to significant public attention in the U.S.A. in the late 1930's over deaths caused by the use of elixirs of sulphonamides where di-ethyleneglycol was used as the solubilising agent. This led to the U.S. Government introducing safety clauses into existing drug laws and, in 1956, to set up the Federal Agency we know today as the F.D.A. Other Governments followed suit, and the process was rapidly accelerated by the teratogenic side-effects in children born to mothers that had taken thalidomide as a sedative/ hypnotic during the early period of their pregnancies.

The effect of all this on the time-scale and cost of drug development can be seen from the increased volume of regulations covering safety clearance studies (Fig. 31), the volume of paperwork now submitted compared with some years ago, and the time-lag between first publication and marketing (Fig. 32). The whole process of drug discovery and development to a marketable product now takes between 10 and 15 years and costs of up to $60M per new product have been cited (vide, Wall Street Journal, April 1980).

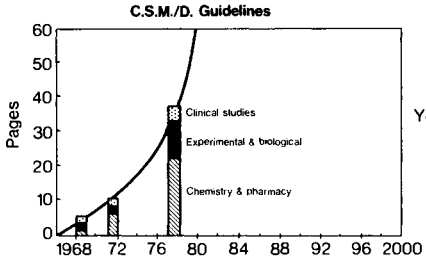


Fig. 31

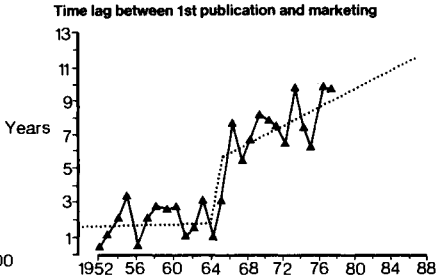


Fig. 32

This lengthening time-scale and ever-increasing cost have a number of implications for the development of new drugs which are affecting, and will increasingly affect, the work of the medicinal chemist and other scientists involved:

1. The time-scale now means that any new drug we discover in our research laboratories today is unlikely (unless it is an anticancer drug or a drug useful in a rare disease situation) to be widely available to patients before the early to mid 1990's. How, therefore, do we decide on research to be carried out today for drugs that will be useful and commercially viable 10 years hence? This must be a factor that would deter newcomers to the field and, for established Companies, the easiest decision is to carry on doing what one is already doing and hope to at least keep ahead in those fields in which one already has a particular position or expertise. This obviously does not encourage real innovation and the breaking of new ground in drug discovery but leads to the increasing tendency to join the next available pharmaceutical bandwaggon that happens to pass by.

2. The time-scale also means that more than half the patent lifetime of the novel compound will have expired by the time any return on the R & D investment is obtained. Since it also takes up to 5 years of marketing before maximum returns can be expected, a Company is only just beginning to get a

reasonable return on its R & D investment when the patent expires and other Companies are free to market the product, subject of course to their being able to satisfy the local Registration and Safety requirements. The case for further extensions of patent life, or some other form of investment protection, is very strong and it is of interest that a move in this direction is under discussion in the Congress of the U.S.A.

3. The cost of new drug development means that most research-based Companies concentrate on those areas of medicine where there is an obvious and substantial demand for treatment. Bacterial infection, hypertension, depression, virus disease, cancer, inflammatory disorders, etc., are all well-covered by industrial research efforts. There is no incentive, currently, for industrial Companies to carry out research into the so-called "minority diseases", for example, disseminated sclerosis, ulcerative colitis, Crohn's disease, bone diseases such as osteoporosis, muscular dystrophy, and many others, some if not all of which should be susceptible to drug treatment. Nor is there any incentive for Companies to invest significant research effort into tropical diseases such as leprosy, malaria, toxoplasmosis, bilharzia, etc., in view of the low prices that could be charged for such drugs. These problems will require special attention and possibly Government or other financial support, since normal commercial returns cannot be expected.

4. Since the pharmaceutical industry does not have benevolent philanthropists supporting it, the increasing costs of drug development have to be reflected in the prices to be charged for new products. Consequently these become a significant part of the cost of health treatment, and since new drugs for the most part are manufactured and supplied by privately-owned Companies, these then become a prime target for Government economies and price restrictions. There still exist, I am told, some Governments even in Europe whose pricing policies preclude the recovery of research investment costs as part of the essential cost of new disease treatment.

The late Sir Derrick Dunlop, at the formal opening of new laboratories at our Brockham Park research centre in 1972, cautioned about "killing the goose that has laid the golden pharmaceutical egg". I can only repeat this warning. If the patients at present treated with modern drugs were to be deprived of such treatment I hesitate to think of the chaos and cost that would ensue to Health Services, since many more patients would require hospitalisation and skilled attention on a 24-hours-a-day basis.

Thoughts for the future

I would not wish to end this review on too gloomy a note. I do sincerely believe that the prospects for discoveries by medicinal chemists are as good, if not better, today than they were 50 years ago providing that we tackle the problems of drug design on a broad front from structure/activity relationships in the laboratory to effectiveness and useful side-effects in the clinic. This will involve increasingly close cooperation between chemists, biologists, metabolic biochemists, clinical pharmacologists and clinicians, if we are to make the most effective use of the large sums of money that must be invested in this work.

Although some therapeutic areas are already well covered by effective compounds, for example, bacterial infection, hypertension, general inflammatory conditions, local anaesthetics, etc., there is an urgent need for more effective compounds for rheumatoid conditions, atherosclerosis, thrombosis (post-operative death), migraine, cancer, virus disease, central nervous system disorders of older patients (senile dementia), and there is much exciting work progressing in most of these areas.

New avenues of research will also open up from the work now escalating in biotechnology (Fig. 33). Although this will perhaps not initially require too much help from synthetic organic chemistry, like the antibiotic story, it will I am sure lead to molecular manipulation of bioengineered molecules to improve their effectiveness, safety, bioavailability, stability, etc. etc.

Pharmaceutical applications of Genetic manipulation and Biotechnology

1. Ready availability of amino acids and peptides
   - Neuropeptides
   - Insulin
   - Interferon
2. Availability of immunoglobulins -antibodies
3. Pure viral proteins (antigens) improved vaccines
4. Availability of enzymes
   - -as chemical reagents
   - -as pharmaceuticals (urokinase)
   - -as diagnostic tools
5. Blood factors
6. Improved antibiotic production (novel antibiotics)
7. Plant cell culture - Steroid precursors

Fig. 33

Finally, I believe that outside of our laboratories we must all do what we can to inculcate into Government civil servants and, perhaps more particularly, the mass media, a proper appreciation of what the medicinal chemist and the pharmaceutical industry has achieved in the past and can and will achieve in the future given the confidence, support, and of course the financial backing, that we need.

ACKNOWLEDGMENT

The author would like to acknowledge the ingenuity and skill of the many scientists in industry and academia who have contributed over many years to the development of the medicinal substances mentioned in this review. He would also like to acknowledge the useful information abstracted from the following publications:

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2'-Fluoro-arabinosyl Pyrimidine Nucleosides: Chemistry, Antiviral, and Potential Anticancer Activities

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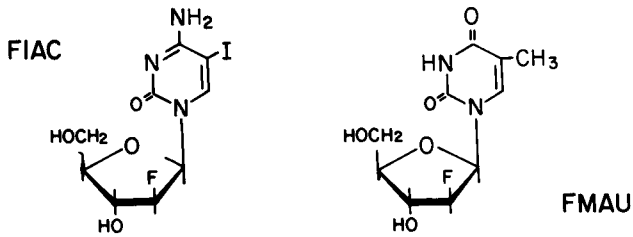
ABSTRACT

A series of 2'-fluoro-5-substituted-arabinofuranosyl-cytosines and -uracils were synthesized. Two of these, FIAC and FMAU, were found to be very potent and highly selective against herpes simplex virus (HSV) types 1 and 2 at very low drug levels. Cytotoxicity to uninfected Vero or human fibroblast cell proliferation was minimal. The selectivity of FIAC against HSV versus its low cytotoxicity against Vero cells is shown to be due, at least in part, to a virus-specified thymidine kinase. Structure-activity studies demonstrate that the 2'-fluoro substituent in the up (arabino) configuration is essential for this potent antiviral activity. Substitution of the 2'-fluoro group by chloro or bromo reduces the antiviral potency. FIAC is also active in a plaque reduction assay against herpes zoster virus at concentrations of 0.01 μM and against cytomegalovirus plaque formation at 0.1 μM. In vivo studies in mice inoculated with 20 LD_{50} of HSV-1 show that FIAC and FMAU are effective, the latter giving 60% cures at dose levels as low as 1 mg/kg/day x5. The selective cytotoxicity of FIAC against human tumor cell lines but not against normal human cells is discussed.



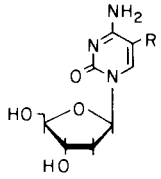
KEYWORDS

FIAC; FMAU; 2'-Fluoro-5-substituted-arabinosylpyrimidines; arabinofuranosyl-pyrimidine nucleosides; anti-herpesvirus agents; anti-herpes zoster virus agent; effects against cytomegalovirus; effects against human cancer cell lines.

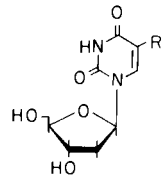
As part of our program in the design and synthesis of compounds of potential value as anticancer or antiviral agents, we undertook several years ago the synthesis of a new class of nucleosides--namely, 2'-halogenated-2'-deoxy-arabinofuranosyl-cytosines and their corresponding -uracils. We describe herein our studies with some of these derivatives, especially with the 2'-fluorinated-5-substituted analogs which offer promise as effective antiviral agents.

The rationale for these undertakings was based in part on the studies of Cooper (1973), Schildkraut (1975) and Greer (1975) who showed that 5-bromo-(BrdC) and/or 5-iodo-2'-deoxycytidine (IdC) (Fig. 1) inhibit the replication of herpes simplex virus (HSV) as effectively as their corresponding 2'-deoxyuridine analogs (BrdU and IdU). Their studies demonstrated that these deoxycytidine analogs are significantly less toxic to uninfected cells than are 5-iodo-(or 5-bromo)-2'deoxyuridine (IdU or BrdU) apparently as a result of a virus-induced pyrimidine nucleoside kinase. This enzyme is required to convert the 5-halogenated-2'-deoxycytidines to their 5'-nucleotides prior to being converted to the corresponding deoxyuridylates.

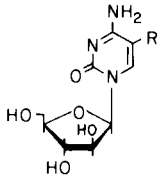
Fig. 1



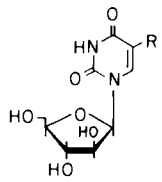
IdC, R = Iodo
BrdC, R = Bromo



IdU, R = Iodo
BrdU, R = Bromo



5-substituted-ara-C's



5-substituted-ara-U's

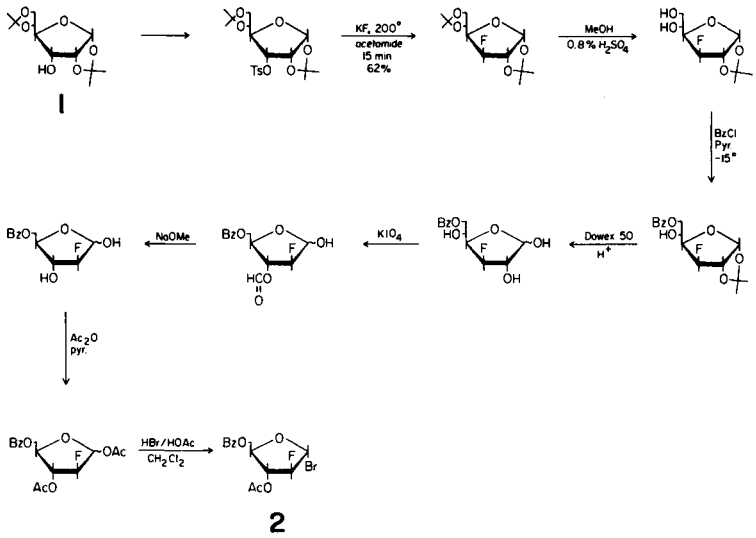
1-β-D-Arabinofuranosylcytosine (ara-C, Fig. 1, R = H), a potent anticancer drug (Talley and co-workers, 1967), also inhibits the multiplication of several DNA viruses in cell culture (Buthala, 1964). Therapeutic trials of ara-C in herpes infections in animal models were not encouraging because its therapeutic to toxic ratio approached unity (Lauter, 1974). Although arabinofuranosyluracil (ara-U, R = H), and some of its 5-alkylated derivatives have been shown to exhibit anti-herpesvirus activities (see De Clercq and Torrence, 1978; Prusoff and Ward, 1976, for reviews), the more attractive of these is arabinosylthymine (ara-T, R = CH_3) which was active against HSV types 1 and 2, as well as against equine herpesvirus (Gentry and Aswell, 1975). Subsequently, Aswell and Gentry (1977) found that 5-methyl-ara-C (R = CH_3) is also active against herpesvirus-infected cells in which

deoxycytidine deaminase is present, indicating that this nucleoside serves as an intracellular donor of ara-T that is phosphorylated to its nucleotide. 5-Halogeno-ara-C derivatives have also shown anti-herpesvirus activity in culture and were also active against experimental herpes keratitis in rabbits (Renis, 1967; Fox, 1966).

From the above-mentioned data, it is obvious that the nature of the substituent at C-5 of the pyrimidine nucleosides shown in Fig. 1 is an important factor in the determination of biological activity. Since activity is noted for both arabino- as well as for 2'-deoxyribo-pyrimidine nucleosides, the C-2' substituent must also play a role. We, therefore, undertook the synthesis of a series of 5-substituted 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)pyrimidines as potential antiviral agents (Watanabe and co-workers, 1979).

Based upon many reports from our laboratory (Fox, 1969; Fox and Watanabe, 1969) and others on pyrimidine nucleoside transformations, it is clear that the direct introduction of a halogeno function in the 2'-"up" (arabino) position would be difficult, if not impossible, because of neighboring group participation by the 2-carbonyl function of the pyrimidine moiety in any displacement reactions. We developed a 9-step synthesis (Reichman and co-workers, 1975) (Fig. 2) of a

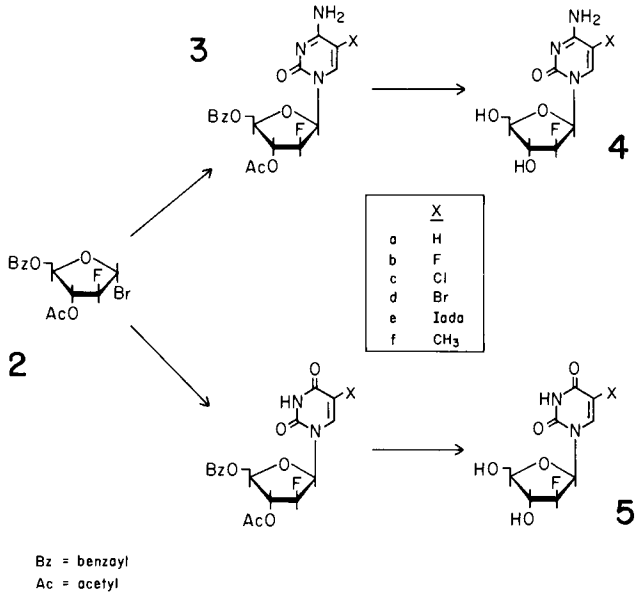
Fig. 2



suitably protected 2'-deoxy-2'-fluoro-arabinofuranosyl bromide (2), a key sugar intermediate, from readily available di-isopropylidene-α-D-allofuranose (1). This synthesis is amenable to large-scale preparations. The syntheses of various 5-substituted derivatives of 2'-fluoro-ara-C was achieved (Watanabe and associates, 1979) by condensation of the key 2'-fluoro-arabinofuranosyl bromide (2) mentioned above with appropriately substituted trimethylsilylated cytosines (Fig. 3) to afford blocked nucleosides (3) which were then de-protected with methanolic ammonia to nucleosides (4). In similar fashion, condensation of halogenose (2) with trimethylsilylated uracils followed by removal of blocking groups with methanolic ammonia gave the 2'-fluoro-ara-U nucleosides (5). The synthesis of the

thymine nucleoside, 2'-fluoro-5-methyl-ara-U, [FMAU, 5f, Fig. 3] is more conveniently prepared (Watanabe, Su, Fox, unpublished) by treatment of 4f with aqueous acetic acid at reflux temperature.

Fig. 3

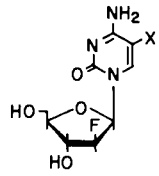
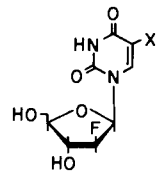


The capacity of these 2'-fluoro-5-substituted-arabinosyl nucleosides [compounds 4 and 5 (a → f)] to suppress replication of HSV type 1 (strain 2931) in monolayers of Vero cells was determined at our Institute (see Watanabe and co-workers, 1979). At 10 μM, almost all of these 2'-fluoro nucleosides showed 99.9% or better suppression of viral replication. The 5-bromo analog (4d) and FMAU were close seconds to 2'-fluoro-5-iodo-ara-C [FIAC, 4e] which was consistently the most potent in these in vitro studies. FIAC gave >90% reduction of the HSV-1 titer in infected Vero cells even at 0.01 μM. Cytotoxicity studies by Lopez and co-workers (1980) were carried out using Vero (monkey) cells which were used also for the antiviral studies. A comparative summary of the antiviral activities of these compounds (expressed as ED_{90}) along with their cytotoxicity toward the uninfected host Vero cells (ID_{50}) is shown in Fig. 4. In terms of the therapeutic indices (ID_{50}/ED_{90}), FIAC emerges as the most potent and selective in this class thus far, followed by 2'-fluoro-5-bromo-ara-C and FMAU. It is noteworthy that 2'-fluoro-5-iodo-ara-U (FIAU, 5d), the deamination product of FIAC, also exhibits good antiviral activity. Against uninfected human fibroblasts (strain WI-38), the concentration of FIAC yielding a 50% reduction of cell proliferation over a 4-day period was ~4 μM. The cytotoxicities using Vero cells were generally similar to those developed earlier with mouse leukemic cells (Lopez, 1980).

The importance of the 2'-fluoro substituent for the anti-HSV-1 activity is emphasized by the data in Table 1 (Watanabe and co-workers, 1979). 2'-Fluoro-5-iodo-ara-C (FIAC, 4e) is much more effective than 5-iodo-2'-deoxycytidine (IdC) or arabinosyl-5-iodocytosine (ara-IC) which indicates that the 2'-fluoro

Fig. 4

Suppression of HSV-1 Replication in Monolayers of Vero Cells by 2'-Fluoro-Arabinosyl-Cytosines and -Uracils

Nucleoside	X	Anti-viral Activity ED_{90}* (μM)	Cytotoxicity ID_{50}† (μM)	Therapeutic Index ID_{50} / ED_{90}
	H	0.03	0.05	1.7
	F	.03	.23	7.7
	Cl	.32	1.1	3.4
	Br	.035	6.7	191
	I	.01	6.7	670
	H	0.32	30	94
	F	.02	0.07	3.5
	Cl	.2	1.0	5
	Br	.08	.7	8.8
	I	.04	.7	17.5
	CH_3	.04	1	25

* ED_{90} = effective dose to inhibit HSV-1 replication by 90 %.

† ID_{50} = dose necessary for 50 % inhibition of growth of Vero cells.

substituent confers better antiviral activity to these 5-iodocytosine nucleosides than does a 2'-hydroxyl or a 2'-hydrogen substituent. A similar conclusion is warranted from a comparison of the 5-iodouracil nucleosides [ara-5-iodouracil (ara-IU), IdU, and FIAU (5e)]. In the thymine series, 2'-fluoro-5-methyl-ara-U (FMAU) is clearly more effective against HSV-1 than is ara-T.

Studies were undertaken in order to focus more closely on structural features essential for these antiviral activities (Lopez and co-workers, unpublished) (see Table 2). To establish the importance of the "up" (arabino) configuration for the 2'-fluoro substituent in FIAC, the 2'-fluoro-5-iodo-ribo-C isomer (2'-fluoro in the "down" configuration) was synthesized (Chu, Watanabe, Fox, unpublished). FIAC was at least 1000x more effective in suppressing HSV-1 replication in Vero cells than the ribo isomer, indicating quite clearly that the 2'-fluoro substituent in the "up" configuration is a requirement for augmentation of the antiviral effect of these compounds.

Preliminary studies have also been carried out to assess the relative merits of a 2'-fluoro versus a 2'-chloro halogen in these compounds (Table 2). The appropriate 2'-chloro sugar analog of halogenose 2 (Fig. 3) had been reported (Ritzmann and co-workers, 1975) from our laboratories. Condensation with appropriate 5-substituted cytosines or thymine afforded the 2'-chloro-arabinosylpyrimidines (Klein, Su, Fox, unpublished) which were examined for their anti-HSV-1 activity along with the corresponding 2'-fluoro analogs. As noted in Table 2, the 2'-chloro analogs demonstrated less anti-HSV-1 activity (and less cytotoxicity).

TABLE 1

COMPARISON OF ANTI-HERPESVIRUS ACTIVITY OF CERTAIN 2'-FLUORO NUCLEOSIDES WITH THEIR CORRESPONDING NON-FLUORINATED ARABINO AND/OR 2'-DEOXYRIBO ANALOGS

NUCLEOSIDE	ANTIVIRAL ACTIVITY* IN μM				
	0.01	0.1	1.0	10	100
ara-C	-	-	+	+++	++++
2'-F-ara-C	-	-	+	+++	++++
ara-Ic	-	-	+	+++	ND‡
IdC	-	-	+	+++	ND
2'-F-5-Iodo-ara-C [FIAC]	+	++	++++	+++++	+++++
Ara-IU	-	-	-	-	+++
IdU	-	-	-	-	++++
2'-F-5-Iodo-ara-U [FIAU]	-	+	+++	++++	+++++
ara-T	-	-	-	+++	+++++
2'-F-5-Me-ara-U [FMAU]	-	+	+++	+++++	ND

* % Reduction of HSV-1 Titer

(-) = <90 +++ = >99.9 ‡ Not Done

\+ = >90 ++++ = >99.99

++ = >99 +++++ = Complete obviation

These results suggest that the more potent anti-herpesvirus activity is probably associated with the 2'-fluoro analogs.

Studies by Lopez and co-workers (1980) with 3 additional HSV-1 strains and two HSV-2 strains indicated (Table 3) that FIAC was about equally active against all of these strains. The ED_{90}'s for these HSV-1 strains ranged from 0.0025 to 0.0116 μM; the ED_{90}'s for the HSV-2 strains were 0.0126 and 0.0044.

When compared with other anti-herpesvirus compounds currently in clinical use (Fig. 5), FIAC was found to be active at much lower concentrations and to be less toxic than ara-C, IdU and ara-A (Lopez, 1980). In a direct comparison with acycloguanosine (ACG), a recently reported compound demonstrating excellent antiviral activity in vitro and in vivo with low toxicity (Elion and co-workers, 1977; Schaeffer, 1978), FIAC was found to be effective at concentrations 6 times lower and to be toxic at 1/10th the concentration of acycloguanosine (acyclo-G), suggesting that (in these in vitro studies) FIAC has a therapeutic index slightly lower or about the same as that of acyclo-G. At higher drug concentrations, FIAC was 60x more active against HSV-1 than acyclo-G.

The selectivity of FIAC against HSV-1 and HSV-2 versus its low cytotoxicity with Vero cells was shown (Lopez and associates, 1980) to depend, at least in part, on the virus-specified thymidine kinase (TK). This conclusion was derived from studies utilizing an HSV-1 mutant lacking this enzyme (HSV-TK⁻). FIAC was found to be active against a variety of TK^+ strains of HSV-1 (with ED_{90}'s of about 0.01 μM) but inactive against an HSV-TK^- mutant strain (ED_{90} ~78 μM). Thus, compared with the wild-type strain of virus (TK^+), the TK^- strain was ~8000-fold less susceptible to FIAC. FIAC appears to be phosphorylated preferentially by this virus-induced enzyme which would account, in significant measure, for the selectivity of its antiherpes activity. In agreement with this possibility,

TABLE 2

COMPARISON OF ANTI-HERPESVIRUS ACTIVITY OF 2'-FLUORO-ARA- vs -RIBO-C AND 2'-FLUORO vs 2'-CHLORO-ARA-C'S AND -ARA-U'S†

Nucleoside	Antiviral Activity in μM				
	0.01	0.1	1.0	10	100
2'-F-5-Iodo-Ara-C [FIAC]	+	++	+++	+++++	+++++
2'-F-5-Iodo-Ribo-C	-	-	-	+	++
[FIAC]	+	++	+++	+++++	+++++
2'-Cl-5-Iodo-Ara-C	-	+	++	+++	++++
2'-F-5-Bromo-Ara-C	-	+	++	+++++	ND**
2'-Cl-5-Bromo-Ara-C	-	-	+	+	+
2'-F-5-Methyl-Ara-C	-	-	+	++	++
2'-Cl-5-Methyl-Ara-C	-	-	-	-	-
2'-F-5-Methyl-Ara-U [FMAU]	+	++	+++	+++++	+++++
2'-Cl-5-Methyl-Ara-U	-	-	-	-	-

preliminary data derived with cytosols of HSV-1-infected and uninfected Vero cells indicate that the virus-specified enzyme phosphorylates FIAC much better than does the enzyme from uninfected Vero cells (Kreis, 1980).

Though FIAC may be viewed structurally as an ara-C analog, its antiviral activity is not reversed by deoxycytidine (Table 4). The minimal toxicity exhibited by high doses of FIAC against uninfected Vero cells (or against human fibroblasts) is reversed by equimolar concentrations of deoxycytidine. The antiviral activity

TABLE 3

CAPACITY OF FIAC TO INHIBIT REPLICATION OF VARIOUS STRAINS OF HSV-1 AND HSV-2 IN VERO CELL MONOLAYERS

HSV Type	Strain	ED_{90}* (μM)
1	Patton	0.0065
	HFEM	.0116
	MacIntyre	.0025
	2931	.009
2	333	0.0126
	G-strain	.0044

Fig. 5

COMPARISON OF ANTI-HSV-1 (2931) ACTIVITY OF FIAC
WITH VARIOUS OTHER ANTIVIRALS

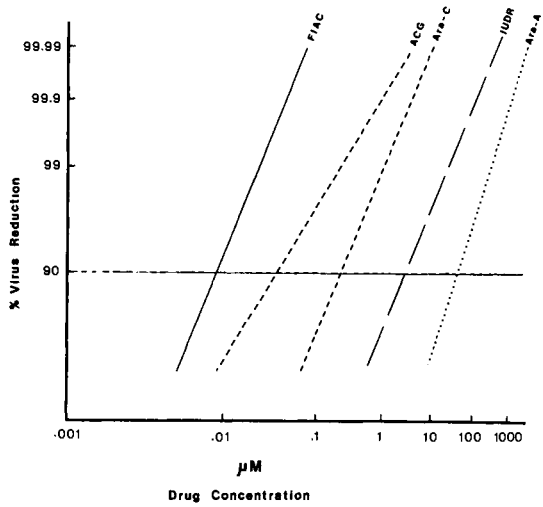


TABLE 4

REVERSAL STUDIES WITH FIAC IN VERO CELLS UNINFECTED AND INFECTED WITH HSV-1

	Antiviral Effect ED_{50}* (μM)	Uninfected Cell Cytotoxicity ID_{50}** (μM)
FIAC	.004	2
FIAC + Equimolar dCtd	.005	33
FIAC + 10 Equiv. dCtd	.007	ND
FIAC + Equimolar dThd	.006	1.8
FIAC + 10 Equiv. dThd	.06	‡

* ED_{50} = concentration required for 50% plaque reduction in vero cells.

** ID_{50} = concentration required for 50% growth inhibition of these cells in a 4-day assay.

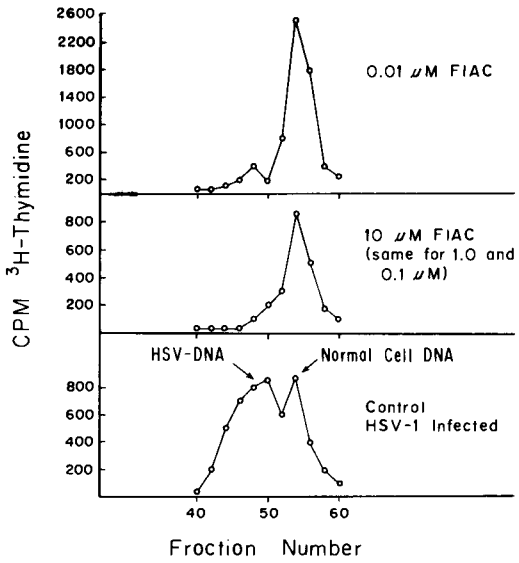
‡ = thymidine toxicity precluded determination.

of FIAC can be reversed in part only by high concentrations of thymidine. [By contrast, the anti-HSV activity of ara-T is readily reversed by equimolar concentrations of thymidine.] These observations (Lopez and co-workers, 1980) suggest that, if necessary, the in vivo toxicity (if any) of large doses of FIAC might be reversed by simultaneous infusion of deoxycytidine. Since the natural nucleoside, deoxycytidine, had no adverse effects on the anti-HSV-1 activity of FIAC, such treatment should not compromise any potential antiviral effect. Moreover, the observation that the low level of toxicity found with normal cells (but not the antiviral effect) could be reversed by deoxycytidine indicates that the former effect is probably mediated by a different mechanism than the latter. It is quite possible that this low level cytotoxicity might depend on phosphorylation of FIAC by a cellular deoxycytidine kinase. This possibility is supported by the finding (Chou and co-workers, 1979) that FIAC demonstrated virtually no cytotoxicity to a mutant mouse tumor cell line (a P815 subline resistant to ara-C) which lacked the deoxycytidine kinase as compared to the parent cell line which had the enzyme and was sensitive to the high dose toxicity.

The selectivity of FIAC against virus-infected Vero cells was studied in greater detail (Lopez, unpublished) using the procedure of Drach and Shipman (1977). In this study (Fig. 6), HSV-1-infected Vero cells were incubated with ^{3}H-thymidine, and the acid insoluble radioactivity (DNA) was separated by centrifugation in cesium chloride gradients (lowest panel) into cellular and viral DNA. The amount of ^{3}H-thymidine incorporated in the acid insoluble fractions was measured in the presence or absence of FIAC. Treatment of HSV-1-infected Vero cells with as little as 0.01 μM FIAC inhibited viral DNA replication by 90%, and 0.1 → 10 μM FIAC completely inhibited viral DNA synthesis. This inhibition of viral DNA

Fig. 6

Capacity of FIAC to Suppress Selectively HSV-1 DNA Synthesis



synthesis was shown to be highly selective since cellular DNA synthesis was not suppressed even at a concentration of 10 μM FIAC. The amount of FIAC required to suppress cellular DNA incorporation of ^{3}H-thymidine by 50% was 3000-fold greater than that required to suppress by 50% the incorporation of label into viral DNA. The high order of selectivity observed within this population of infected cells suggests that this selectivity was not due to the thymidine kinase (all cells had the enzyme) and may be due to virus-specified DNA polymerase.

Herpes zoster virus (HZV) infection of cells has also been reported to be accompanied by the expression of a new, probably virus-specified, thymidine kinase (Ogino and co-workers, 1977). Experiments were therefore undertaken to determine whether FIAC was also effective against HZV. When the Ellen strain of HZV and WI-38 (human fibroblasts) cells were used, FIAC at 0.01 μM inhibited plaque formation by 50% (Lopez and co-workers, 1980).

Cytomegalovirus (CMV) infection of susceptible cells does not result in a viral specific pyrimidine nucleoside kinase (Estes and Huang, 1977). Unlike HSV-1, HSV-2 and HZV, CMV induces the expression of increased concentrations of cellular thymidine kinase. Studies, nevertheless, were undertaken (Lopez and associates, 1979) to determine whether CMV was also susceptible to FIAC. In these preliminary studies, FIAC suppressed CMV plaque formation in the two strains studied (AD-169 and 303 and 1/60th the concentrations cytotoxic for the WI-38 cells (ID_{50} = 4 μM) used in these experiments. Further investigation of this interesting aspect of FIAC activity is needed.

Chou and associates (1980) report that FIAC is deaminated by human leukemic and by monkey kidney (Vero) cells at rates comparable to that for ara-C and further that this deamination is inhibited by tetrahydrouridine. Using [2-^{14}C] FIAC, the incorporation of radioactivity into DNA of uninfected Vero cells is competitively inhibited by deoxycytidine but not by thymidine. In HSV-1 infected versus uninfected Vero cells, this incorporation is enhanced fivefold and may be strongly inhibited by thymidine but only weakly by deoxycytidine. These data indicate that FIAC behaves metabolically like thymidine in HSV-1-infected cells but like ara-C in non-infected and leukemic cells and are consistent with the studies of Lopez and co-workers (1980) mentioned above. In current studies on the metabolic fate of FIAC in mice, Philips and associates (1980) using [2-^{14}C] FIAC report that FIAC and its deaminated metabolites (FIAU or partly FMAU) are incorporated into DNA.

In a comprehensive in vitro study by De Clercq and associates (1980) on compounds which have been reported to exert a selective inhibitory effect on the replication of HSV, FIAC was found to be among six compounds emerging as most potent and selective anti-herpesvirus agents (Table 5). Whereas 5-(2-bromovinyl)-2'-deoxyuridine emerged as the most potent anti-HSV-1 compound (see Table 5) among these nucleosides (averaged for 11 strains of HSV-1), acycloguanosine and FIAC were the most potent against HSV-2 (as averaged for 7 strains).

Based upon the in vitro data shown in Tables 1 and 2, two candidates, FIAC and FMAU, were selected for in vivo experiments (Fig. 7). In these studies (Lopez and co-workers, unpublished) mice were inoculated IP with 20 LD_{50}'s of HSV-1 (strain 2931) and treated beginning 12 hours later with 100, 10, or 1 mg/kg/day administered IP for 5 days. Though both drugs were potent, FMAU clearly was more effective in vivo than FIAC. Sixty percent of the mice treated with only 1 mg/kg/day x5 of FMAU survived the infection, and all mice treated with 10 or 100 mg/kg/day survived. By comparison, 40% of the mice treated with 100 mg/kg/day, and 20% of those treated with 10 mg/kg/day x5 of FIAC also survived.

These results suggest (as has been also pointed out by others) that in vivo

TABLE 5

SUSCEPTIBILITY OF STRAINS OF HSV-1 AND HSV-2 TO INHIBITORY EFFECTS OF ANTIHERPES COMPOUNDS IN PRIMARY RABBIT KIDNEY CELL CULTURES*

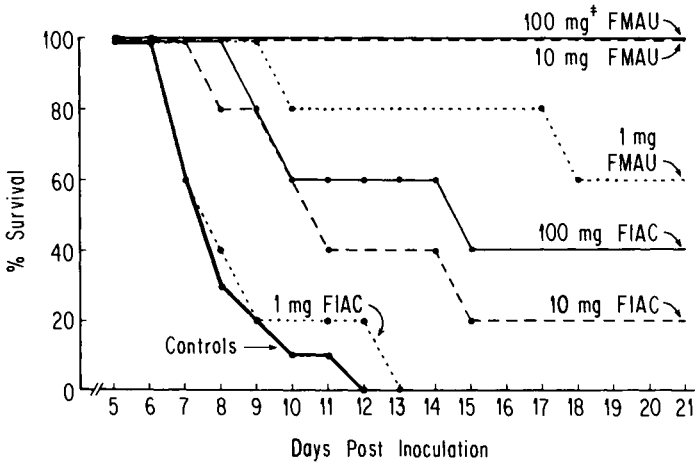
Nucleoside	Average for 11 HSV-1 strains ID_{50} (μg/ml)	Average for 7 HSV-2 strains ID_{50} (μg/ml)
E-5-(2-bromovinyl)-2'-deoxyuridine	0.008	1
E-5-(2'-iodovinyl)-2'-deoxyuridine	.012	2
5-vinyl-2'-deoxyuridine	.018	0.1
2'-fluoro-5-iodo-ara-C [FIAC]	.017	.05
Acycloguanosine	.04	.04
5-iodo-2'-deoxycytidine [IdC]	.06	.3

* Data taken from De Clercq et al.

studies may not always parallel in vitro results. FMAU, in vitro, was about equal in potency against HSV-1 as FIAC, yet it is far superior to it in vivo. Limited toxicity studies with FMAU in mice have also been done (Lopez, Burchenal, unpublished). Mice have been treated with 400 mg/kg/day x5 FMAU without demonstrating weight loss or other adverse effects. The in vivo toxicity of FMAU is probably comparable to that of FIAC.

Fig. 7

Survival or BALB/C Mice Inoculated with 20 LD_{50} of HSV-1 (strain 2931) and then Treated with FIAC or FMAU



* Mice given a 5-day course of drug starting 12 hrs. after inoculation IP with virus

‡ Concentrations in mg/kg/day administered IP

Since a number of studies have shown that human tumor cells express "fetal" thymidine kinase activity, which has less stringent substrate specificity (Taylor and co-workers, 1972; Stafford and Jones, 1972; and Lee and Cheng, 1976), studies were undertaken to determine whether FIAC was cytotoxic to human tumor cells in culture. In Table 6 are listed 8 cell lines tested with FIAC in a 4-day assay for cytotoxicity. These human tumor cells were found (Burchenal and associates, 1979) to be more susceptible to the cytotoxic effect of FIAC than were the normal human cell lines or mouse leukemia cell lines. Moreover, the low dose cytotoxicity against human tumor cells is not reversed by deoxycytidine, but the high dose toxicity to normal cells and mouse leukemia cells is reversed by equimolar concentrations of deoxycytidine. This selectivity of FIAC against the human tumor cell lines may be due to a nonspecific nucleoside kinase which is more efficient than the specific deoxycytidine or thymidine kinases of normal human or mouse leukemic cells in phosphorylating FIAC. If this enzymatic difference operates *in vivo*, it could afford a significant therapeutic advantage.

In conclusion, our results thus far with FIAC and FMAU attest to the promise which this new class of compounds, the 2'-fluoro-5-substituted-arabinosylpyrimidine nucleosides, offer as a source of potent and selective anti-herpesvirus agents. Indeed, the syntheses of newer analogs in this class continues in our laboratories. Preclinical toxicological, pharmacological and biochemical studies are underway at our Institute which are geared to increase our understanding of their possible mode(s) of action and for eventual clinical trials.

TABLE 6

SELECTIVE ACTIVITY OF FIAC AGAINST HUMAN TUMOR CELLS BUT NOT AGAINST NORMAL HUMAN CELLS OR MOUSE TUMOR CELLS IN CULTURE

		ID_{50} (μM)
HUMAN TUMOR CELL LINES		
1. K562	(MYELOGENOUS LEUKEMIA)	0.1
2. HeLa	(CARCINOMA)	0.1
3. KB	(CARCINOMA)	0.1
4. Molt-3	(ALL)	0.5
5. Namalva	(Burkitt's lymphoma)	0.1
6. RAJI	" "	0.5
7. DAUDI	" "	0.1
8. P3HR1	" "	0.5
NORMAL HUMAN CELL STRAINS		
1. WI-38	(LUNG FIBROBLAST)	2-10
2. PHA-STIMULATED LYMPHOCYTES		10
3. FS-4	(SKIN FIBROBLASTS)	10
MOUSE TUMOR CELL LINES		
1. L5178Y		25
2. P815		35

ACKNOWLEDGEMENT

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Nucleoside Antibiotics. Mode of Action

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ABSTRACT

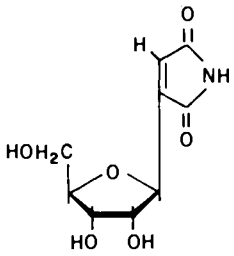
Over sixty nucleoside antibiotics have been described and their chemical structures are now elucidated. According to their mode of action these compounds can be classified in the following groups:
1. Inhibitors of nucleic acid synthesis (including: 1a inhibitors of nucleotide synthesis, 1b inhibitors of purine and pyrimidine interconversions, 1c inhibitors of nucleotide utilization and 1d nucleoside analogs that are incorporated into polynucleotide chains replacing natural nucleosides); 2. Inhibitors of protein synthesis (including: 2a purine nucleoside antibiotics and 2b pyrimidine nucleoside antibiotics); and 3. Miscellaneous nucleoside antibiotics (including: 3a inhibitors of adenosine deaminase, 3b inhibitors of cell-wall synthesis and 3c cyclic-AMP phosphodiesterase inhibitors).
The different groups of inhibitors are described, considering particularly the important group of protein synthesis inhibitors which specifically act on the acceptor site of the peptidyl transferase center. Within this group the purine nucleoside antibiotics puromycin, lysylaminoadenosine and homocitrullylaminoadenosine are included. They act as acceptor substrates in the peptide bond formation reaction. On the other hand, a number of purine (including nucleocidin and antibiotic A201A) and pyrimidine nucleoside antibiotics (including amicetin, bamicetin, oxamicetin, plicacetin, norplicacetin, gougerotin and analogs, blasticidin S and anthelmycin) which are known to inhibit peptide bond formation are not active as acceptor substrates in this reaction. The chemical structure requirements for inhibitory action by the antibiotics is described.

KEYWORDS

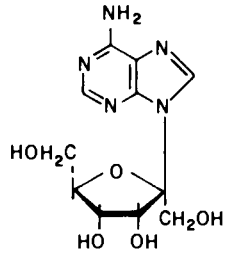
Protein synthesis inhibitors; puromycin; purine nucleoside antibiotics; pyrimidine nucleoside antibiotics; nucleoside antibiotics.

INTRODUCTION

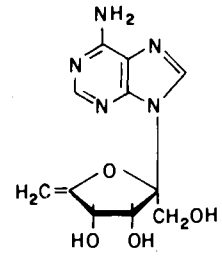
More than sixty nucleoside antibiotics have been described in the last thirty



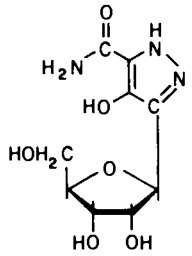
Showdomycin
(group 1a)



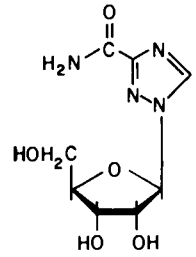
Psicofuranine
(group 1b)



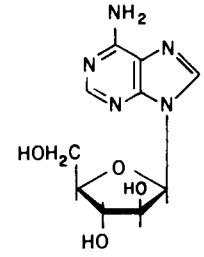
Decoyinine
(group 1b)



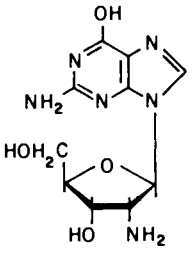
Pirazofurin
(group 1b)



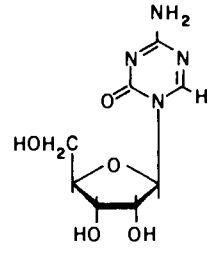
Ribavirin
(group 1b)



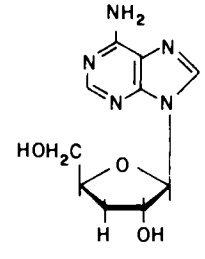
9-β-D-arabinofuranosyl-adenine (Ara-A). (group 1c)



2'-Aminoguanosine
(group 1d)

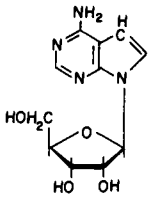


5-Azacytidine
(group 1d)

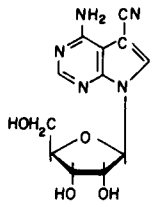


Cordycepin
(3'- deoxyadenosine)
(group 1d)

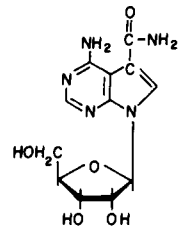
Fig. 1. Chemical structures of thuringiensin and some nucleoside antibiotics of groups 1 and 3



Tubercidin
(group 1d)



Toyocamycin
(group 1d)



Sangivamycin
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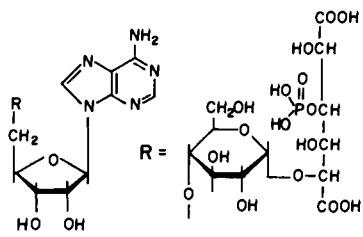
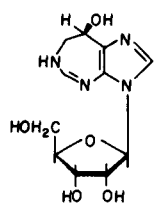
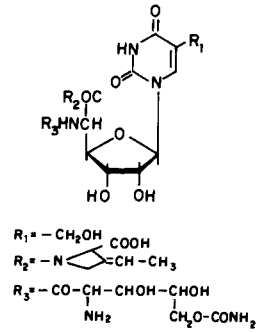


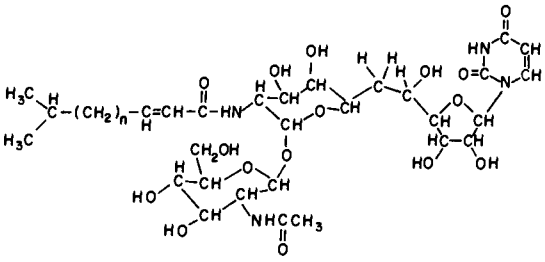
Thuringiensin
(group 1d)



Coformycin
(group 3a)

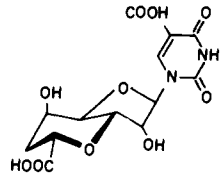


Polyoxin A
(group 3b)



Tunicamycins
(group 3b)

A n = 9
B n = 10
C n = 8
D n = 11



Octosyl acid A
(group 3c)

years. Within this group of antibiotics are included some compounds that are useful as either antitumour (tubercidin, 5-azacytidine, ara-A, pyrazofurin) or antiviral (ribavirin) or antifungal (polyoxins) agents and others which are very important tools in biological studies. Indeed the purine nucleoside antibiotic puromycin has been a very useful tool to elucidate the mechanism of peptide bond formation in protein synthesis. Similarly polyoxin A is the most important inhibitor to study chitin synthetase and cell-wall formation in microorganisms having chitin as the main structural component in their wall. These few examples might illustrate the importance of the nucleoside group of antibiotics which have been reviewed recently (Suhadolnik, 1979). However it is very difficult in most cases to correlate structure and activity of the nucleoside antibiotics and to predict their mode of action. Indeed as we will see in the following chapters these inhibitors fall in different categories according to their mode of action. We can widely classify the nucleoside antibiotics according to their mode of action in three categories: 1 Inhibitors of nucleic acid synthesis; 2. Inhibitors of protein synthesis; 3. Miscellaneous inhibitors.

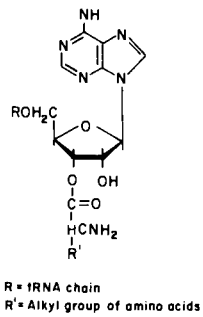
INHIBITORS OF NUCLEIC ACID SYNTHESIS

This is the amplest category of nucleoside antibiotics considering the number of compounds included. Although it might occur in some cases that an inhibitor can interfere with different cellular reactions in the pathway leading to nucleic acid synthesis we can clearly distinguish at least two levels of antibiotic action on this process. The compounds acting at the level of nucleotide metabolism are included in one of these groups and the agents unbalancing the polymerization reactions involved in nucleic acid synthesis are included in the second group. Since three different groups of compounds can be distinguish within the inhibitors acting at the level of nucleotide metabolism we have altogether the following groups: 1a inhibitors of nucleotide synthesis; 1b Inhibitors of purine and pyrimidine interconversions; 1c Inhibitors of nucleotide interconversion; 1d Nucleoside antibiotics that serve as unnatural substrates for the DNA and RNA polymerization reactions leading to the synthesis of nonfunctional nucleic acids. A number of compounds of these four groups are presented in Fig. 1 including also the nucleotide antibiotic thuringiensin. Although the compounds are tentatively classified within one of the four groups this might not be entirely correct since some of the compounds might inhibit more than one reaction and the mode of action of some others is incompletely understood.

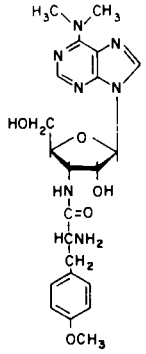
INHIBITORS OF PROTEIN SYNTHESIS

Within this category are included the compounds whose action is best understood at a molecular level and therefore we shall concentrate attention on these agents. According to their chemical structure we can distinguish two groups: 2a Purine nucleoside antibiotics; 2b Pyrimidine nucleoside antibiotics.

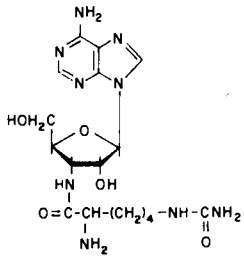
Nucleoside Antibiotics



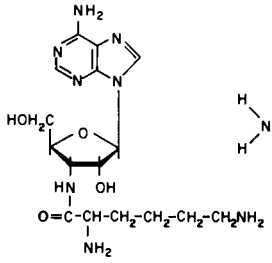
3'end of aminoacyl-tRNA



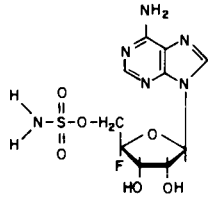
Puromycin



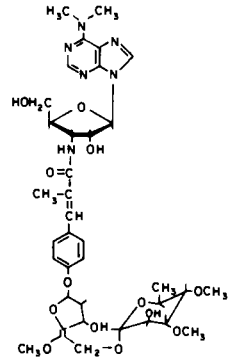
Homocitrullylaminoadenosine



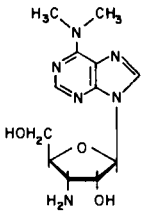
Lysylaminoadenosine



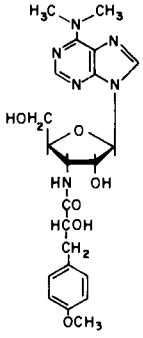
Nucleocidin



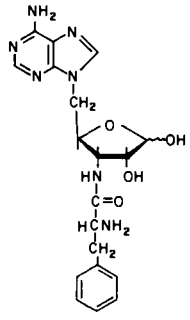
A 201A



Puromycin aminonucleoside



α-Hydroxy-puromycin



"Reversed puromycin"

Fig. 2. Chemical structures of the 3' end of aminocyl-tRNA, purine nucleoside antibiotics that inhibit protein synthesis (puromycin, homocitrullylamino-adenosine, lysylaminoadenosine, nucleocidin and A201A) and related compounds (puromycin aminonucleoside, α-hydroxy-puromycin and "reversed puromycin").

Purine nucleoside antibiotics

The most interesting compounds of this group are puromycin, homocitrullylaminoadenosine, lysylaminoadenosine, nucleocidin and A201A (Fig. 2). All these antibiotics have very wide inhibitory spectra since they interact with ribosomes from bacteria and higher cells and thus inhibit protein biosynthesis in both prokaryotic and eukaryotic cells. However they differ in their mode of action.

The great importance of puromycin lies in its being the clearest example of an antibiotic inhibitor of translation whose chemical structure is obviously an analog of a component of protein synthesis. Indeed puromycin is an analog of the 3' terminal end of aminoacyl-tRNA (Fig. 2) and therefore interacts with the acceptor site of the ribosomal peptidyl transferase center on the larger ribosome subunit (50S in bacterial and 60S in higher cells ribosomes). However, puromycin lacks that part of the aminoacyl-tRNA molecule for interactions with the ribosome other than with the peptidyl transferase. Hence, the use of the antibiotic provides a simplified method for the study of peptide bond formation in a reaction in which the $-NH_2$ group of puromycin becomes linked to the C-terminal end of f-Met- or the peptidyl-group ("puromycin reaction") (Fig. 3). The product of the puromycin reaction (f-Met- or peptidyl-puromycin) is unable to take part in the next step of protein synthesis. However, all the evidence indicates that the formation of a peptide bond between puromycin and the f-Met- or peptidyl-group takes place by the same mechanism as peptide bond formation in protein synthesis. Polyphenylalanyl-tRNA, polylysyl-tRNA, f-Met-$tRNA_F$, and Ac-Phe-tRNA are suitable donor substrates in the puromycin reaction. The terminal CACCA-, AACCA-, ACCA- and CCA-Met-f from f-Met-$tRNA_F$ (and also CACCA-Phe-Ac and CACCA-Leu-Ac) undergo a ribosome-catalyzed reaction with puromycin to yield f-Met-puromycin in a simplified system known as the "fragment reaction" (Fig. 3). The reaction requires only the larger ribosomal subunit, monovalent and divalent cations, and either methanol or ethanol, and takes place with the corresponding f-Met- or Ac-Phe- or Ac-Leu-oligonucleotides from the 3' terminal end of either f-Met-tRNA or Ac-Phe-tRNA or Ac-Leu-tRNA respectivety. The limiting factor in this reaction is the affinity of the substrate for the donor site of the peptidyl transferase; thus peptide bond formation also takes place with the donor substrates CA- and A-Met-f, but a high concentration of A-Met-f is required since its affinity for the active center is very low. Thus the donor site of the peptidyl transferase appears to have specific sites not only for adenosine but also for cytidylic acid, since this compound increases the binding of A-Met-f considerably (over 400 %) (Vázquez, 1979).

The structural requirement for activity as an acceptor substrate like puromycin has been studied by using a number of puromycin derivatives and aminoacyl-nucleosides. It has been concluded in these studies that (a) "reversed puromycin" (Fig. 2) is inactive, (b) both the diaminonucleoside and the amino acid moieties of puromycin are required for its activity, (c) the nucleoside extra amino group has to be at the 3' position and (d)

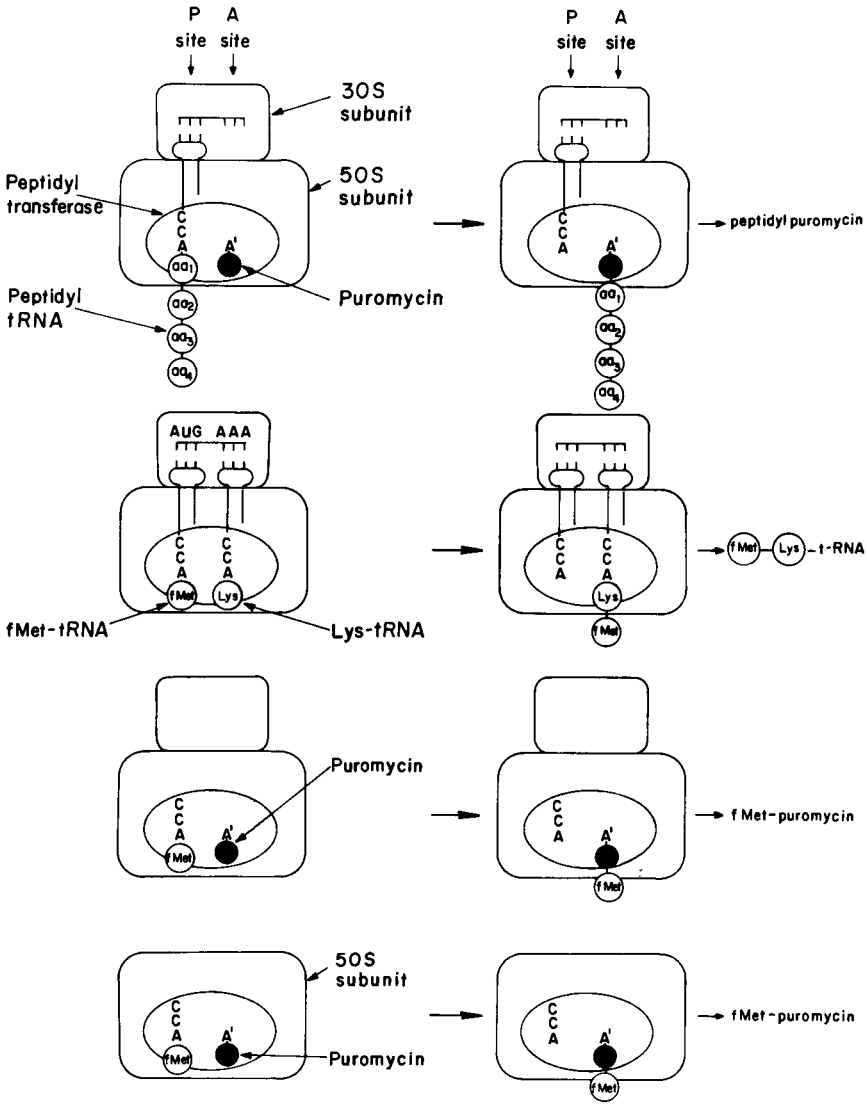


Fig. 3. Schematic representation of a number of model systems to study peptide bond formation by bacterial ribosomes. From top to bottom are represented different systems using as donor substrates either peptidyl-tRNA or f-Met-tRNA or CCA-Met-f and as acceptor substrates either puromycin or lysyl-tRNA. Finally it is represented the most simplified system using 50S ribosomal subunits, CCA-Met-f as a donor substrate and puromycin as an acceptor substrate.

higher activities are always obtained with amino acids of L-configuration whem compared with the same amino acids of D-configuration (Nathans, 1967; Vázquez, 1979; Suhadolnik, 1979). In most cases a hydrophobic amino acid moiety appears to be required but the synthesis of an active puromycin analog with a hydrophilic amino acid has been reported. (3'-N-[S-(6-hydroxyhexyl)-L-cysteinyl]puromycin).(Vince and Fong, 1978). The dimethyl groups, the methoxyl group, the furanosyl oxygen and the 5' hydroxyl group of puromycin are not required for biological activity. In cell-free systems L-Phe-, L-Tyr-, and L-Leu-adenosine equal puromycin in activity as acceptor substrates in the peptide bond formation reaction (Vázquez, 1979). Indeed the weak interaction of (^{3}H) pyromycin with the acceptor site of the peptidyl transferase center is inhibited by Phe-adenosine (Fernández-Muñoz and Vázquez, 1973).

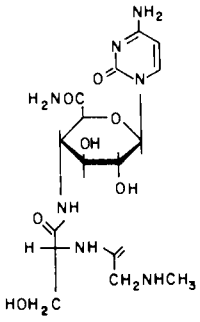
The puromycin derivatives α-hydroxy- (Fig. 2) and demethoxy-α-hydroxypuromycin are of special significance. These compounds lack the α-amino group of puromycin and are therefore unable to form a peptide bond, but they form an ester bond with the f-Met moiety of f-Met-tRNA or CACCA-Met-f in a reaction catalyzed by the peptidyl transferase center of the ribosome (Fahnestock and others, 1970). On the other hand the antibiotics nucleocidin and A201A, lacking of the α-NH_2 or α-hydroxy group (Fig. 2), are inhibitors of protein synthesis but have no acceptor activity in the peptide bond formation reaction (Suhadolnik, 1979).

In order to identify the ribosomal components involved in the interaction with puromycin, affinity labeling studies have been carried out using a number of puromycin analogs and derivatives. Unfortunately the results obtained in these studies appear to be rather contradictory and do not clarify the interaction site of puromycin. Different ribosomal proteins or even the ribosomal RNA can be labelled depending on the experimental conditions or the puromycin analogs used (Table 1).

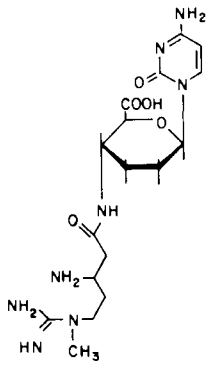
TABLE 1 Labelling of ribosomal components with puromycin and puromycin derivatives

Method	Ribosomal components which are labeled	Reference
Affinity labeling with N-iodo-[2-^{14}C] puromycin	Proteins L6 and S18	Pongs and others (1973)
Photo affinity labeling with (^{3}H)puromycin	Ribosomal RNA and proteins L23 and S14	Cooperman and others (1975)
Photoaffinity labeling with an aryl azide analogue of puromycin	Proteins L11 and S18	Nicholson and Cooperman(1978)
Affinity labeling with a puromycin derivative	The sequence G U C C Cp of 23S RNA (C is a modified C)	Eckermann and Symonds (1978)
Photoaffinity labeling with a puromycin analogue	Proteins L6, L13, L18, L22 and L25	Krassnigg, Erdmann and Fasold (1978)

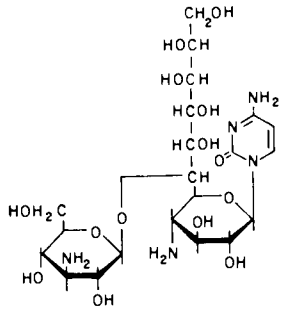
L proteins are from 50S and S proteins are from 30S subunits



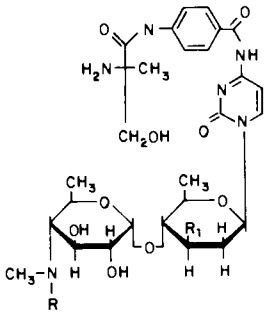
Gougerotin



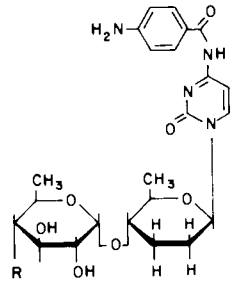
Blasticidin S



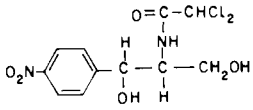
Anthelmycin
(synonym hikizimycin)



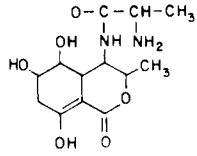
Amicetin (R = CH_3 ; R_1 = H)
Bamicetin (R = H ; R_1 = H)
Oxamicetin (R = CH_3 ; R_1 = OH)



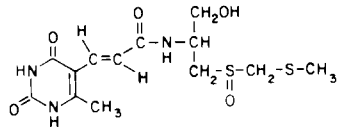
Plicacetin (R = $(CH_3)_2N$)
Norplicacetin (R = CH_3HN)



Chloramphenicol



Actinobolin



Sparsomycin

Fig. 4. Chemical structures of pyrimidine nucleoside antibiotics that inhibit protein synthesis (gougerotin, blasticidin S, anthelmycin, amicetin, bamicetin, oxamicetin, plicamicetin and norplicacetin) and other protein synthesis inhibitors that act on the acceptor site of the peptidyl transferase center (chloramphenicol, actinobolin and sparsomycin).

Puromycin binds to the 50S subunits of bacterial ribosomes. Furthermore a number of proteins of the 50S subunits can be dispensed without affecting the interaction of puromycin. Indeed it was shown in early studies that some protein deficient particles (γ-cores) can be prepared from 50S subunits by isopycnic centrifugation in CsCl solutions of decreasing Mg^{++} concentrations (Monro and others, 1969). The cores contain 23S and 5S RNA but lack proteins L1, L6, L7, L10, L12, L15, L16, L25, L31 and L33 and have reduced amounts of L5, L9, L11, L18, L20, L27, L28 and L30 (Nierhaus and Montejo, 1973). However γ-cores bind (^{3}H)puromycin to the same extent as 50S ribosome subunits (Table 2).

There are few reports concerning the mode of action of homocitrullylaminoadenosine (Guarino, Ibershof and Swain, 1970) and lysylaminoadenosine (Suhadolnik, 1979), but all the evidence so far obtained suggests that these antibiotics act in a similar way to puromycin in cell-free extracts of both Escherichia coli and rat liver.

TABLE 2. (^{3}H)Puromycin binding to 50S ribosomal subunits and derived cores

Conditions	(^{3}H)Puromycin bound (pMoles/tube)
Expt. 1	
50S	1.80
γ-cores	2.10
$SP_{50-\gamma}$	0.87
γ-cores + $SP_{50-\gamma}$	2.44
Expt. 2	
50S	2.24
γ-cores	2.04
$SP_{50-\gamma}$	0.11

(^{3}H)Puromycin binding to 50S subunits, 50S-derived γ-cores and the split protein fraction released in the obtention of γ-cores from 50S subunits ($SP_{50-\gamma}$) was studied by equilibrium dialysis (Fernández-Muñoz and Vázquez, 1973). Concentration of (^{3}H)-puromycin was 10^{-6} M. 50S subunits were added when indicated at 12.5 mg/ml. γ-cores (15mg/ml) and the corresponding amount of the $SP_{50-\gamma}$ fraction were added when required. Equilibrium dialysis was carried out for 10 hours at 4ºC. In Experiment 1 the indicated particles or protein fractions were preincubated, prior to the equilibrium dialysis, in mM Tris-HCl buffer, pH 8.0, containing 20 mM Mg, 0.1 M NH_4 and 3 mM 2-mercaptoethanol for 90 min at 50ºC. No such preincubation was performed in Experiment 2. Experiments 1 and 2 were carried out simultaneously with the same batch of ribosomes.

Pyrimidine nucleoside antibiotics

The 4-aminohexosyl cytosine antibiotics amicetin and analogs (bamicetin, oxamicetin, plicacetin, norplicacetin) gougerotin and analogs blasticidin S and anthelmycin (Fig. 4) are included in this group. All of them have a very wide inhibitory spectrum acting on ribosomes and hence blocking peptide bond formation in systems from bacteria, mitochondria, chloroplasts and higher cells.

All these pyrimidine nucleoside antibiotics inhibit peptide bond formation in the puromycin and the fragment reaction assays. They are very effective inhibitors of (^{3}H) puromycin, CACCA-(^{3}H)Phe, (^{3}H) blasticidin S and (^{3}H) gougerotin binding to the ribosome and it has been therefore concluded that antibiotics of this pyrimidine nucleoside group interact with the acceptor site of the peptidyl transferase center. However none of the antibiotics of this group have acceptor activity in peptide bond formation, unlike it occurs with puromycin. A cooperative effect on substrate and antibiotic binding to the acceptor and donor sites has been repeatedly observed. Thus peptidyl-tRNA bound to the donor site enhances chloramphenicol and sparsomycin interation with the acceptor site. Conversely thiamphenicol, sparsomycin and the pyrimidine nucleoside antibiotics enhance substrate binding to the donor site (Vázquez, 1979). The 4-aminohexosyl cytosine antibiotics might act on overlapping rather than identical sites since ribosomes from a yeast mutant resistant to anthelmycin are sensitive to the other antibiotics of the group (González, Santamaría, Vázquez and Jiménez, 1980).

In the series of amicetin analogs it has been shown that the amino acid seryl is an important structural feature for their activity and the absence of this residue results in antibiotics (plicacetin and norplicacetin) with a decreased activity. Moreover cytosinine, the nucleoside portion of blasticidin S is devoid of activity (Cerna, Rychlik and Lichtenthaler, 1973). On the other hand on the bases of studies with a large series of gougerotin analogs it has been concluded that (a) the 3'-hydroxyl group is very important for inhibitory activity, (b) the dipeptide in the 4' position is required for activity, (c) the introduction of a double bond between the 2' and 3' positions of the carbohydrate moiety causes a marked decrease in activity but there is no such a decrease if, in addition, the sarcosyl-D-seryl moiety is replaced by an ε-N-methyl-β-L-arginyl side chain, (d) replacement of the -D-seryl residue with -L-seryl or an increased number of other aminoacyl residues tested considerably decreases the activity and (e) replacement of the carboxamide group at position 5' with a hydroxymethyl function results in reduced activity. Furthermore the replacement of either the -D-seryl or the sarcosyl-D-seryl residue by a number of aminoacyl-residues does lead to any compound with acceptor activity in the reaction of peptide bond formation (Coutsogeorgopoulos and others, 1975).

Considering the results obtained with different antibiotics of the group it has been proposed that four structural features are required for the inhibitory activity of 4-aminohexosyl cytosine antibiotics: (a) a similar spacial arrangement of one oxygen and three nitrogen atoms in the nucleoside moiety, (b) the presence of an amide bond between the respective

aminoacyl residues and either 4-aminohexuronic acid (gougerotin and blasticidin S) or 4-aminobenzoic acid (amicetin and bamicetin) moieties, (c) hydrogen bonding of the -OH...OC-type in the seryl moiety of gougerotin, amicetin and bamicetin and of the $-NH_2....O=C$ type in the arginine moiety of blasticidin S and (d) a terminal N-methylamino group (Lichtenthaler and Trummlitz, 1974). However the anthelmycin molecule is a very active antibiotic (González, Vázquez and Jiménez, 1979) and only fits the first structural requirement since it is even deprived of any amino acid moiety (Fig. 4). On the other hand the antibiotic sparsomycin appears to have a mode of action similar to the 4-aminohexosyl cytosine antibiotics (Vázquez, 1979) and cross-resistance of mouse blasticidin S-resistant cell lines to puromycin and sparsomycin has been reported (Kuwano, Takenaka and Ono, 1979) although sparsomycin has a very different structure (Fig. 4). This statement is also valid for actinobolin (Fig. 4) although it has a much reduced activity. Chloramphenicol (Fig. 4) also interacts with the acceptor site of the peptidyl transferase center of bacterial ribosomes but is devoid of activity on ribosomes of eukaryotic cells; moreover chloramphenicol does not overlap with the binding sites of the 4-aminohexosyl cytosine antibiotics (Vázquez, 1979).

Miscellaneous Inhibitors

There are an increasing number of nucleoside antibiotics than cannot be included within the first two categories of inhibitors and therefore are grouped within a third category including miscellaneous inhibitors. Within this group of compounds are of particularly relevant importance: 3a inhibitors of adenine deaminase such as coformycin; 3b inhibitors of cell-wall synthesis including polyoxin A that inhibits chitin synthetase and the tunicamycins that inhibit peptidoglycan and glycoprotein synthesis; 3c inhibitors of cyclic-AMP phosphodiesterase in which octosyl acid A is included (Fig. 1) (Suhadolnik, 1979).

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Antitumor and Antiviral Activity of N^6-Hydroxyadenine Nucleosides and Related Derivatives

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ABSTRACT

The first member of the series, N^6-hydroxyadenosine (HAPR), exerted an antitumor activity in mouse leukemias and underwent clinical trials. A toxicity was observed, due to enzymatic hydrolysis by adenosine deaminase. 9-β-D-Arabinosyl-N^6-hydroxyadenine (ara-HA) had an inhibitory effect in herpes simplex virus type 1 (HSV-1) replication, but it was also toxic. The 6-nitroso purine nucleosides of the ribosyl and arabinosyl series were obtained by oxidation of the corresponding N^6-hydroxyadenine (HAP) derivatives. Nitrosation of N^6-hydroxyadenine led to the N^6-(nitroso)hydroxyamino derivative which, in contrast to its base, was inactive. Nitrosation of 9-β-D-ribofuranosyl-N^6-(1-methylhydrazino) or N^6-methylaminopurine gave N^6-(methylnitroso)adenosine which exerted growth inhibitory activity in mouse leukemia. The use of adenosine deaminase inhibitors, erythro-9(2-hydroxy-3-nonyl)-adenine (EHNA) and 2'-deoxycoformycin (2'-DCF) resulted in an increased antitumor and antiviral activity of the ribosyl and arabinosyl N^6-hydroxyadenines, but they were ineffective in blocking the toxic effects in primates. These results prompted the study of new derivatives with substituents at C-2, as it is known that 2-substituted adenosines are resistant to enzymatic attack. In fact, the 2-amino (AHAPR) and 2-fluoro (FHAPR) N^6-hydroxyadenosines were less affected by enzymatic hydrolysis than the unsubstituted nucleosides. The N^2,N^6-dihydroxyaminopurine ribonucleoside (DHAPR) was effective against mouse leukemia and colon 38 tumor. All these N^6-hydroxyadenosines had potent anti-HSV-1 activity in vitro. The biological effect of substituents in the nucleosides depends on their structure, being more effective those with N-OH function on the purine nucleus.

KEYWORDS

HAPR; ara-HA; DHAP; DHAPR; mono- and disubstituted 9-ribosylpurines; monosubstituted 9-arabinosylpurines; antitumor agents; antiviral agents; enhancing effect of adenosine deaminase inhibition.

INTRODUCTION

We have developed adenosine derivatives which exert antitumor and antiviral activity The first derivative of the series, N^6-hydroxyadenine, was expected to have biological activity since it can be regarded as an analog of adenine and hypoxanthine,

and also because of the extraordinary chemical reactivity of the N-hydroxy group.

The line of development of some of the N^6-hydroxyadenine nucleosides can be visualized in a modified scheme (Fig. 1) originally drawn by Burchenal (1975) in which the successive development of antimetabolites is shown starting from 2,6-diaminopurine, the first purine derivative found to exert antitumor effect:

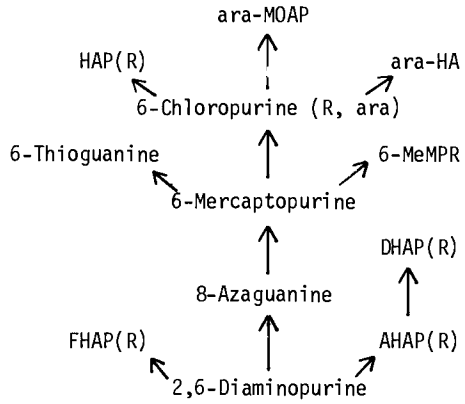
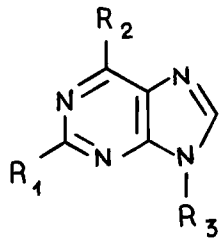


Fig. 1. Synoptic development of some antitumor and antiviral purines and nucleosides (R = ribosyl; ara = arabinosyl).

In addition to the N-hydroxy function, other substituents from which we prepared purine and purine ribonucleoside analogs are: methoxyamino, methylhydroxyamino, 1-methylhydrazino, nitroso, methylnitroso, nitroso-isopentenyl, nitrosobenzyl, nitroso-hydroxyamino, hydrazo, azo, glycinyl and morpholino derivatives (Fig. 2).



R_1 = H, F, NH_2, NHOH, Cl

R_2 = SH, SCH_3, NHOH, $NH(OCH_3)$, $N(OH)CH_3$, CH_3-N-NH_2, NO, (NO)N-OH, $N(NO)CH_3$, (NO)N-isopentenyl, (NO)N-benzyl, NH-NH, N=N, glycinyl, morpholino

R_3 = H, ribosyl

Fig. 2. Some purines and ribonucleoside derivatives.

N^6-hydroxyadenine Nucleosides

We have synthesized and evaluated purine nucleosides of the ribosyl and arabinosyl series with one or two substituents in the purine moiety.

RIBOSYL NUCLEOSIDES

The purine derivative, N^6-hydroxyadenine (HAP) which can be considered as an analog of adenine and hypoxanthine (Fig. 3), exerted a moderate growth-inhibitory effect against L1210 mouse leukemia (Giner-Sorolla and Bendich, 1958). This agent, however, presented the inconvenience of its sparing solubility in water and instability in aqueous solutions or suspensions due to its oxidation to the nitroso derivative. Consequently, the 9-β-D-ribofuranosyl derivative, N^6-hydroxyadenosine (HAPR), was synthesized to investigate the effect of the introduction of the ribosyl group on the chemotherapeutic index of N^6-hydroxyadenine. The expected greater solubility of N^6-hydroxyadenosine and its metabolic product would minimize the nephrotoxicity encountered with its parent base, adenine (Bendich and co-workers, 1950; Giner-Sorolla and co-workers, 1966a; Philips and co-workers, 1952), and enhance its therapeutic index. Adenine is a toxic substance, devoid of any therapeutic activity, but adenosine and N^6-methyladenosine exert an inhibitory effect on the appearance of spontaneous tumors (Strong and co-workers, 1973). The toxicity of N^6-hydroxyadenine was attributed (as in the case of adenine) to its metabolic oxidation to 2,8-dihydroxyadenine which deposits in the kidney tubules as determined by large-dose administration to rodents (Giner-Sorolla, 1966).

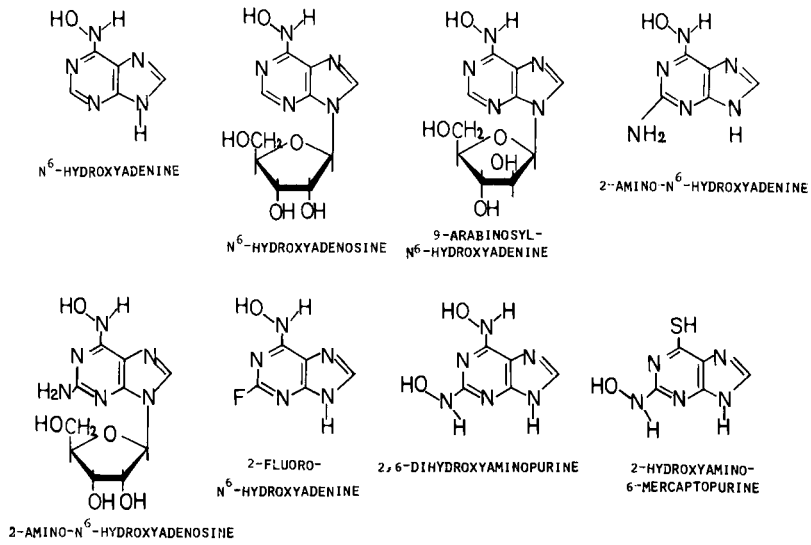


Fig. 3. Main N^6-hydroxyadenine derivatives.

Early studies by Burchenal and co-workers (1967) showed that HAPR was considerably more active than the parent base (HAP) against a broad range of experimental tumors, including mouse leukemias L1210 and P815. HAPR was found by Bloch and

co-workers (1966) to exert an inhibitory effect on S180 ascites tumors *in vitro* and *in vivo*. The most valuable properties of HAPR were its effectiveness on mouse leukemia lines resistant to conventional chemotherapeutic agents and the remarkable synergistic effect when administered in combination with other chemotherapeutic agents. In a P815 mouse leukemia line resistant to 6-mercaptopurine, at a dosage of 60 mg/kg of HAPR, the increase in life span (ILS) was 170% and at 300 mg was 286%. In sublines of leukemia P815 resistant to methotrexate, vincristine, actinomycin and arabinosyl cytosine, HAPR gave also significant increases in life span. More significant results were obtained in the studies of combination therapy with HAPR, thus, in L1210 mouse leukemia at 100 mg/kg qdx10 HAPR produced an ILS of 87%, while with 6-mercaptopurine (6-MP) at 10 mg/kg, the ILS was 34%. When both agents were used in combination at the same dosage, the ILS reached 326% (Burchenal and co-workers, 1967).

N^6-Hydroxyadenosine and 9-β-D-ribofuranosyl-6-methylmercaptopurine (6-MeMPR) are active against a line of L1210 mouse leukemia resistant to 6-MP, which has lost the pyrophosphorylase pathway for conversion of 6-MP to the active 6-MP nucleotide (Brockman, 1965). This fact indicates that N^6-hydroxyadenosine does not reach the active nucleotide level by the pyrophosphorilase route. The effectiveness with which adenosine, but not adenine, blocks the effect of N^6-hydroxyadenosine on L1210 could be attributed to competition for adenosine kinase. In addition, the fact that the HAPR-resistant line of L1210 mouse leukemia is also cross-resistant to 6-MeMPR, but not to 6-MP or thioguanine, suggests that HAPR is also phosphorylated by adenosine kinase and that resistance found to HAPR should be attributed to a loss in this adenosine kinase activity (Burchenal and co-workers, 1967).

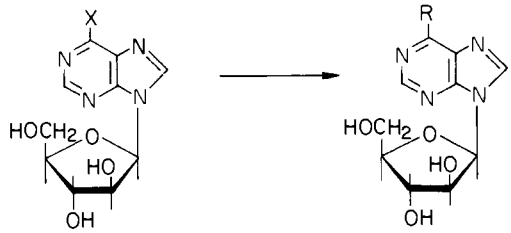
In view of the data discussed above and after preliminary pharmacological studies, clinical trials were carried out at Memorial Hospital with N^6-hydroxyadenosine in patients with advanced inoperable neoplastic diseases (Krakoff and Dollinger, 1969; Dollinger and Krakoff, 1975). Although no apparent cardiac or vasopressor effects were found by its administration, hemolysis was observed in all cases, presumably caused by the hydroxylamine released by the adenosine deaminase which is present in the erythrocytes (Agarwal and co-workers, 1975). In this rapid hydrolytic cleavage, N^6-hydroxyadenosine is converted into inosine. Enzymatic hydrolysis of HAPR has been observed with adenosine deaminase from ox heart (Rockwell and Maguire, 1966), from intestinal calf mucosa, and L1210 mouse leukemia cell extracts (Giner-Sorolla and co-workers, 1977a). It was, therefore, suggested by Philips and associates that the enzyme present in erythrocytes had the same hydrolytic activity towards N^6-hydroxyadenosine, leading to the intracellular release of hydroxylamine, which is known to exert hemolytic activity (Kiese, 1966).

Other N^6-hydroxyadenosine derivatives we synthesized were the N^6-methylhydroxyamino and 6-methoxyamino (prepared by nucleophilic displacement of 9-β-D-ribofuranosyl-6-chloropurine with the corresponding amine), N^6-(nitroso)hydroxyadenosine (obtained by nitrosation of HAPR) and methyl(nitroso) derivatives obtained by the nitrosation of N^6-methyladenosine or even in better yields by the oxidation of 6-(1-methylhydrazino)-9-β-D-ribofuranosylpurine with nitrous acid (Giner-Sorolla and co-workers, 1973; Greenbaum and co-workers, 1978). In contrast to the corresponding bases of these nitroso derivatives which showed antitumor activity (Giner-Sorolla and co-workers, 1973), the ribonucleoside derivatives were ineffective.

The corresponding nucleotide to N^6-hydroxyadenosine prepared by treatment of 9-β-D-ribofuranosyl-6-chloropurine 5'-phosphate with ethanolic hydroxylamine, had activity both *in vitro* in several cell lines and *in vivo* against mouse L1210 comparable to that of the nucleoside.

ARABINOSYL DERIVATIVES

In the foregoing discussion we have shown how the introduction of an oxygen in the amino group at C-6 of adenine resulted in derivatives which exhibit antitumor activity. These data stimulated the investigation of an additional structural alteration to be made in the sugar moiety of the corresponding N^6-hydroxyamino, 6-methoxyamino, N^6-methylnitroso, nitrosopurine and other nucleosides (Fig. 4) (Giner-Sorolla, 1969; Giner-Sorolla, 1970; Giner-Sorolla and co-workers, 1973; Giner-Sorolla and Taracido, 1975).



X = Cl, SH, SCH_3, SO_2CH_3

R = $CH_3-N(-OH)-$, $CH_3O-N(-H)-$, $-NHNH_2$,

$CH_3-N(-NH_2)-$, $CH_3-N(-NO)-$

Fig. 4. Synthesis of arabinosylpurine derivatives.

The synthesis of 9-β-D-arabinofuranosyl-N^6-hydroxyadenine (ara-HA) was carried out by ethanolic hydroxylamine treatment (Giner-Sorolla, 1969) of the corresponding 6-chloropurine derivative, prepared by a modification of the method of Reist and co-workers (1962) (Fig. 5). Ara-HA was intended originally to be evaluated as an anticancer agent; it differs structurally from 9-β-D-arabinofuranosyladenine (ara-A) by the presence of an oxygen at the N^6-position. The rationale behind the design of this drug was to synthesize a purine analog resistant to enzymatic deamination and which could thus exert an enhanced antitumor activity. We found tha ara-HA had a slight inhibitory effect in several rodent experimental neoplasms an a low toxicity. The lack of toxicity can be attributed to the fact that ara-HA i not a good substrate for adenosine deaminase in the mouse. In other animal speci we have observed enzymatic hydrolysis of ara-HA, although at a lower rate than th occurring with ara-A.

We evaluated ara-HA as an antiherpesvirus agent because of its close structural relationship to ara-A, its lack of toxicity in rodents, and the known chemical an biological reactivity of the -NHOH function. In addition, the greater water solu bility of ara-HA versus ara-A may be advantageous for its administration.

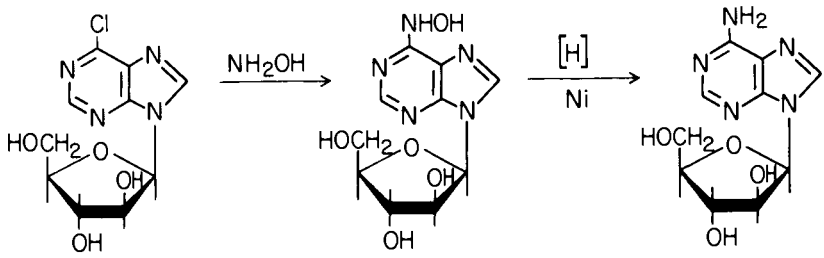


Fig. 5. Preparation of ara-HA and its reduction to ara-A.

We found that ara-HA is a potent inhibitor of herpes simplex virus type 1 (HSV-1) infection in mice. In fact, our comparative in vivo studies in the mouse showed that ara-HA is more effective than ara-A, one of the best currently available anti-herpesviruses. We used for these assays A/J (susceptible strain) mice which were inoculated with 100 LD_{50} of HSV-1 (2931 strain); one day later groups of 10 mice were started on 10-day courses of 200, 50 or 10 mg/kg/day of ara-HA or 200 mg/kg/day of ara-A. All control mice died about 7 days after inoculation. Those receiving 200 mg/kg/day for 10 days survived the HSV-1 infection, while the same dose of ara-A protected only 60% of the mice. (Fig. 6). All the mice that had received 200 mg/kg/day ara-HA survived 28 days post-inoculation at which time they were challenged with 10^5 LD_{50} of HSV-1. All mice survived challenge indicating that they had survived because they were immune to HSV-1. A similar test with ara-A was not possible as all of the treated animals died after 21 days (Lopez and Giner-Sorolla, 1977).

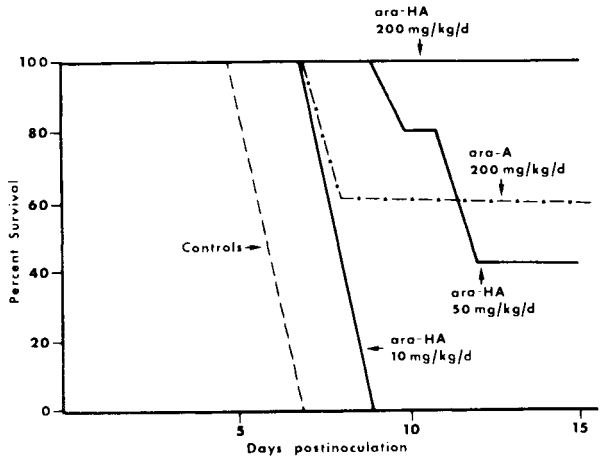


Fig. 6. Effect of arabinosyladenine and arabinosyl N^6-hydroxyadenine on herpesvirus infection in vivo.

ENHANCEMENT OF THE THERAPEUTIC EFFECT OF SOME PURINE NUCLEOSIDES BY ADENOSINE DEAMINASE INHIBITORS

Since the hemolytic effects of N^6-hydroxyadenosine found after its administration to cancer patients could be attributed to the hydroxylamine released by the adenosine deaminase in the erythrocytes, new clinical trials were undertaken at Memorial Hospital in which dypyrimadole (Bunag and co-workers, 1964; Emmons and co-workers, 1965), an adenosine deaminase inhibitory agent, was used in combination with N^6-hydroxyadenosine. This inhibitor gave poor results in relieving the hemolytic effect of the nucleoside. The introduction by Schaeffer and Schwender (1974) of an effective and reversible adenosine deaminase, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) stimulated our interest in the study of its combination therapy with N^6-hydroxyadenosine. Another adenosine deaminase inhibitor of greater potency than EHNA, 2'-deoxycoformycin (2'-DCF) (Woo and co-workers, 1974), has been used by LePage and co-workers (1976) in combination with ara-A in L1210 or L1210 mouse leukemias resistant to ara-C; an increase in survival time was observed in both cases.

We also studied the rate of deamination *in vitro* of N^6-hydroxyadenosine using adenosine deaminase from calf intestinal mucosa and its inhibition by EHNA according to methods previously described (Rockwell and Maguire, 1966; Roth and co-workers, 1964; Schaeffer and Vince, 1965). A 1:100 molar ratio of the inhibitor to HAPR gave complete inhibition; the inhibition was 90% at 1:250 and 70% at 1:500 (Giner-Sorolla and co-workers, 1977b).

The combination of HAPR and EHNA in P815 mouse leukemia cell suspensions showed that N^6-hydroxyadenosine alone at 5 μg, 2.5 μg and 1.25 μg/ml gave inhibition values of 66, 31 and 8%, respectively. When the same experiments were carried out in the presence of 0.3 μg of EHNA, the inhibition rates were 100, 100 and 89%, respectively. *In vivo* screening in mouse leukemia L1210 showed there was a moderate increase of antitumor effect of N^6-hydroxyadenosine by the addition of the adenosine deaminase inhibitor. Thus, HAPR alone at 50 mg/kg qdx5 produced an increase in life span (ILS) of 83% over the controls (Giner-Sorolla and co-workers, 1977b); with EHNA at 5 mg/kg the ILS increased 120%. At 10 mg/kg of HAPR qdx5 the ILS was only 65%; when this dose was combined with EHNA at 0.5 mg/kg, the ILS was 87%. This increase in antileukemic effect should be attributed to the blocking of adenosine deaminase by the inhibitor in parallel to similar combination therapies with arabinosyladenine (Plunkett and Cohen, 1975).

We reported that ara-HA, the N^6-oxidized form of ara-A, had a slight inhibitory effect when administered in experimental rodent tumors (Burchenal, unpublished results). We carried out combination therapy with ara-HA and EHNA or 2'-DCF; no antitumor activity was found in L1210 mouse leukemia at a variety of dose levels. This lack of antitumor effect of ara-HA in combination with an ADI contrasts with that observed by others (LePage and co-workers, 1976; Plunkett and Cohen, 1975) with ara-A and 2'-DCF. It also differs from our results described above with HAPR and EHNA or 2'-DCF.

In contrast to the lack of response of transplanted animal tumors to the combination of ara-HA and adenosine deaminase inhibitors, we found an enhancement of inhibitory effect on herpesvirus replication with this combination. As shown in Fig. 7, all mice inoculated with HSV-1 as described before and treated with ara-HA (50 mg/kg/day) and EHNA (10 mg/kg/day) were alive after 20 days while all the controls died. Only 60% of the animals receiving ara-HA alone (at the same dosage) survived. When ara-HA and EHNA were given at doses of 10 mg/kg/day each, 70% of the treated mice survived. In contrast, when ara-HA was given alone in doses of 10 mg/kg/day only 30% of the animals survived. EHNA alone at the same dose did not affect the survival rate of the mice infected with HSV-1. Similar assays with arabinosyladenine N^1-oxide in combination with EHNA (10 mg/kg/day) showed that at 50

mg/kg/day, 40% of the animals survived the HSV-1 infection; whereas without EHNA the N^1-oxide gave no increase in survival time. In these cases, the enhancement of the effect may be due to the blocking of the hydrolysis caused by adenosine deaminase. Thus, their conversion to 9-β-D-arabinosylhypoxanthine (which is a less active antiviral agent) is prevented. Adenosine N^1-oxide may act by metabolic conversion to ara-HA or ara-A (Giner-Sorolla and Lopez, 1978).

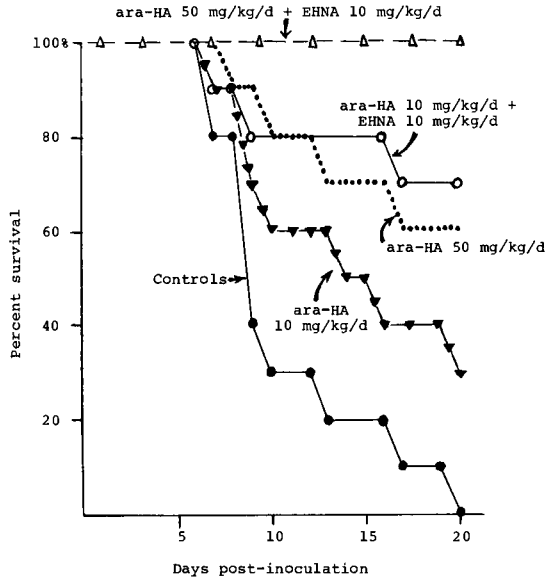


Fig. 7. Effect of EHNA on the antiviral activity of ara-HA.

When HAPR and ara-HA were administered to primates in combination with EHNA or 2'-DCF in a variety of dosage levels, it was found that hemolysis was always present indicating the inability of the adenosine deaminase inhibitors to block the enzymatic hydrolysis (Philips, personal communication). It is for this reason that we studied the effect of substitution at C-2 by several functional groups (NH_2, F, NHOH) as it is known that substituents at this position are resistant to enzymatic deamination (Montgomery and Hewson, 1957).

The oxidation of N^6-hydroxyadenine ribo- and arabinonucleosides with active manganese dioxide led to the corresponding 6-nitroso derivatives. These compounds were designed to serve as precursors of the N^6-hydroxyadenine; they appeared to be unstable and had only some inhibitory effect *in vitro* against L1210 cell culture, but not *in vivo* (Giner-Sorolla and co-workers, 1977a).

2-SUBSTITUTED PURINE NUCLEOSIDES

We have investigated 2-substituted purines (guanine analogs) such as 2-amino-2-N-hydroxyamino and also 2-fluoro-N^6-hydroxyadenine and their 9-ribosyl derivatives (Giner-Sorolla and co-workers, 1966b; Giner-Sorolla and Burchenal, 1971) (Fig. 8).

These derivatives increased the survival time of mice inoculated with L1210 and P815 mouse leukemias to a lesser extent than N^6-hydroxyadenosine, but they were less readily hydrolyzed by adenosine deaminase (Giner-Sorolla and co-workers, 1976). 2-Fluoro-N^6-hydroxyadenosine (FHAPR) was less effective in P815 mouse leukemia than HAPR; it was toxic at dosages of 50 mg/kg. 2-Amino-N^6-hydroxyadenosine (Giner-Sorolla and co-workers, 1966b), when tested against L1210 mouse leukemia at 50 mg/kg qdx5 gave an average survival time (AST) of 36 days versus 9.7 days for the controls.

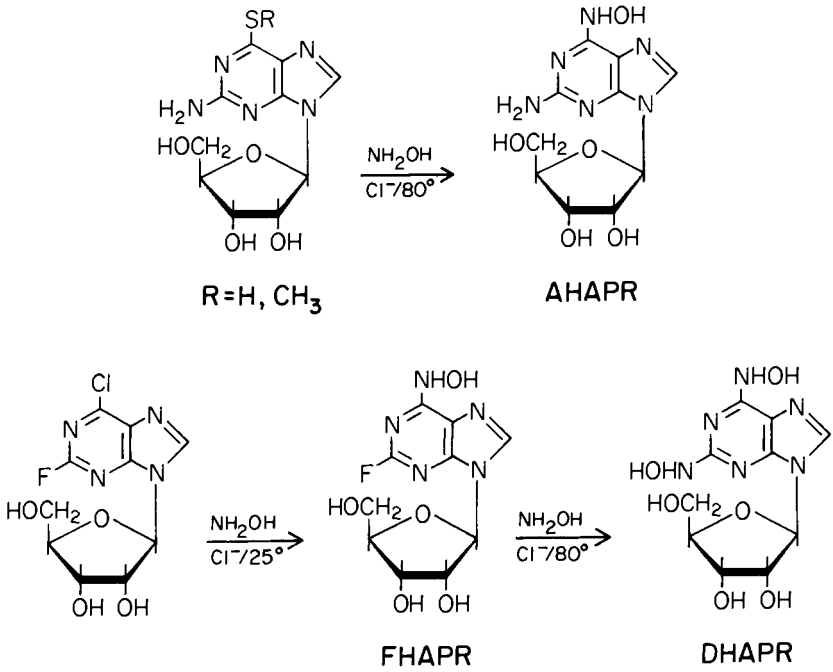


Fig. 8. Synthesis of some substituted N^6-hydroxyadenosine derivatives.

DHAP is the N-oxidized analog of 2,6-diaminopurine, the first purine to exhibit antitumor and antiviral effects (Burchenal and co-workers, 1949). In mouse leukemia, DHAP showed an optimum growth inhibitory effect at 3 mg/kg qdx5 giving a 60% survival after 60 days; at doses above and below 3 mg it was less active (Fig. 9). Since this compound showed toxicity only beyond 300 mg/kg, the loss of activity could be attributed to the immunosuppressive effect which is common to this type of agent (Giner-Sorolla and co-workers, 1968, 1972). The 9-ribosyl derivative of 2,6-dihydroxyaminopurine (DHAPR) was less active against L1210 mouse leukemia, but was found effective against mouse colon 38 tumor and had a greater solubility and less toxicity than DHAP in rodents (Giner-Sorolla and co-workers, 1968); but it is hydrolyzed by adenosine deaminase from human erythrocytes.

An overview of different experiments of the effect of several hydroxyaminopurine derivatives on survival time on mice with leukemia L1210 is in Fig. 10, which shows

the antitumor effect of four N^6-hydroxyadenine derivatives; HAPR, the base (DHAP), its 9-ribosyl nucleoside (DHAPR), and the 2-amino derivative of HAPR (AHAPR), compared with 6-MP. The most active compound, DHAPR, gave at 25 mg/kg/5qd, a 60% survival after 35 days, followed by AHAPR, which gave 40% survival at 50 mg/kg/5qd.

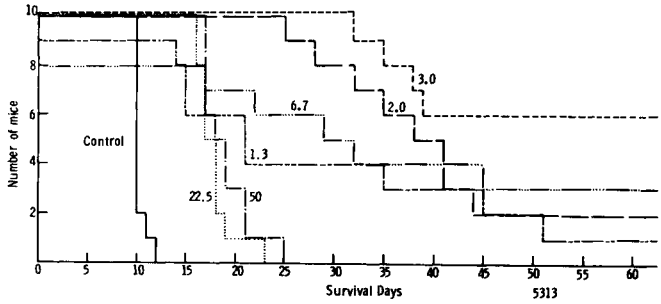


Fig. 9. Activity of different dosages (mg/kg qdx5) of DHAP against leukemia P815.

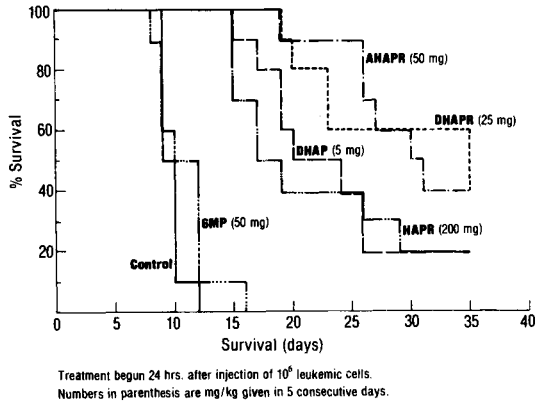


Fig. 10. Effect of several hydroxyaminopurines on survival time of mice with leukemia L1210.

AHAPR exerted a marked inhibitory effect against HSV-1 replication in vitro. These assays were carried out in Vero monolayers infected with 1 plaque-forming unit (PFU) as previously described (Lopez and Giner-Sorolla, 1977). In Table 1 its effect is shown in comparison with other nucleosides.

An effect found in the majority of anticancer drugs is their carcinogenic properties (Weisburger, 1977); antibiotics and alkylating agents are usually the most

potent carcinogens. Antimetabolites also exert carcinogenic effect although to a lesser extent than the above-mentioned groups. Table 2 shows the comparison of anticancer and carcinogenic effects of several adenine derivatives which we have found, in collaboration with Dr. M. N. Teller.

TABLE 1 In Vitro Inhibition of Herpes Simplex Replication by Purine Nucleosides (%)

	ara-HA	ara-A	AHAPR	DHAPR	FHAPR	HAPR
1000 µg/ml	99.8	99.8	-	-	-	-
100 µg/ml	92	96.6	99.8	98	98	91
10 µg/ml	0	58	99.2	98	85	71
1 µg/ml	-	-	80.3	78	71	40

The dosage for a carcinogenic effect is always greater than the therapeutic. In addition, the administration of the agent for carcinogenesis bioassays consisted of repeated doses for several months (Table 2).

TABLE 2 Duality of Anticancer and Carcinogenic Effect of Several N^6-Hydroxyadenine Derivatives

	Dose* (mg/kg qdx10)	ILS* (%)	Total dose** (mg)	%** Tumors
N^6-Hydroxyadenine	200	86	220	60[a]
N^6-Hydroxyadenosine	270	189	250	0[a]
N^6-Methylhydroxy-adenine	100	15	240	75[a]
N^6-Hydroxyadenine 3-oxide	25	50	200	80[a]
	200	120		
N^6-(Methylnitroso)-adenosine	125	45	40	100[b]

[a] = rats; [b] = mice; *Mouse L1210 leukemia; **Carcinogenesis bioassays; ILS = increase in life span.

DISCUSSION AND CONCLUSIONS

The strategy of cancer chemotherapy is based upon the cytostatic effect of drugs with selective toxicity. Among these agents, heterocyclic nucleosides stand out for their marked antitumor and antiviral effect. Their development is based on rational principles: the interference with the supply of purine nucleotides or in the inhibition of certain key enzymes. In this manner, the established control systems that govern nucleic acids affecting growth and cell division are circumvented. The structure-activity relationship in purine antimetabolites usually shows a clear-cut distinction between active or inactive functional or structural substitutions. Although the information gathered relating to anticancer or antiviral activity to a given structural modification has led to the possibility of predicting biological activity, these predictions do not always result in effective therapeutic agents.

Of the different substituents at C-6 and C-2 of the purine nucleus evaluated, only the N-hydroxyamino and to a lesser extent the methoxyamino functions showed tumor inhibitory effect - the N^6-hydroxyamino derivative being active in vivo against herpesvirus (HSV-1) replication in the case of ara-HA and in vitro with the ribonucleosides. The failure of adenosine deaminase inhibitors to block the undesirable effect of the agents could be compensated by a search of derivatives with appropriate substituents at the C-2 or 5'-nucleotides since this type of compound appears to be resistant to enzymatic attack.

The experience obtained in recent years with the development of antimetabolites is contributing towards the search for new drugs with a greater selectivity. The accumulation of data on the structure-activity relationship of new agents from experimental and clinical sources can lead to the rational design of more effective anticancer and antiviral agents.

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Alkylating Nucleosides

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ABSTRACT

A series of N-glycosyl-halomethyl derivatives of 1,2,3-triazole, -pyrazole, -imidazole and -1,2,4-triazole has been prepared as a new type of alkylating agents. The design of these nucleosides as potential antitumor drugs is based on the use of the chemically alkylating benzylic type halide as the active moiety of such compounds. Halomethyl-1,2,3-triazole nucleosides have been prepared by 1,3-dipolar cycloaddition of glycosyl azides to halomethyl acetylenes. Halomethyl-pyrazole, -imidazole and -1,2,4-triazole alkylating nucleosides have been synthesized by glycosylation of the corresponding halomethyl azoles or by generation of the halomethyl group on suitable substituted nucleosides previously formed. The in vitro and in vivo cytostatic activities as well as the mode of action of these alkylating nucleosides have been studied. Several compounds have been effective against ECA tumor and P388 lymphocytic leukemia. Relationships between these activities and certain structural features, such as halogen atom, substituents on the heteroaromatic ring, and nature, size and protecting groups of the sugar moiety are discussed. An alkylating mechanism of action is suggested.

KEYWORDS

Alkylating agents; halomethyl azoles; 1,2,3-triazole nucleosides; pyrazole nucleosides; imidazole nucleosides; 1,2,4-triazole nucleosides; cytostatic activity; HeLa cells; Ehrlich carcinoma ascites; P-388 lymphocytic leukemia.

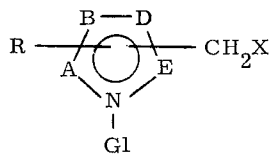
INTRODUCTION

Compounds that are able to alkylate nucleophilic centres of chemical components of the cell have been largely used in connection with cancer chemotherapy. Although this action is not, in general, specific for neoplastic tissue, some alkylating agents, such as merophan or cyclophosphamide have shown specificity against certain tumoral systems (Yount and colleagues, 1967; Carbone and

colleagues, 1969). These and other examples (Sartorelli and Johns, 1975) encouraged us to search for new types of alkylating agents that could be toxic towards a limited range of neoplasms.

Several theoretical approaches have been used in order to increase the specificity of the biological action of alkylating agents (Ross, 1974). One of the most important approaches consists on the attachment of the alkylating group to carrying structures normally involved in cell growth, such as amino acids, carbohydrates, steroids and nucleic acid components, bases, nucleosides and nucleotides. Although the first attempt to combine an alkylating agent, a nitrogen mustard group, with a nucleic acid component, the purine nucleus, did not led to the desired N,N-bis(2-chloroethyl) amine (Hüber, 1956), success was later found in the synthesis of the sulfur mustard analog (Johnston and colleagues, 1958) and of several purines or pyrimidines containing alkylating moieties. Thus, 5-[bis(2-chloroethyl)amino] uracil and its 6-methyl derivative (Dopan) are effective against tumors of the hematopoietic system (Williams, 1963; White, 1961), 5-[[bis(2-chloroethyl)amino] methyl] uridine is active against leukemia (Farkaš and Šorm, 1969), and 9-alkyl and 9-ribofuranosyl derivatives of 6-(1-aziridinyl) purines show activity against adenocarcinoma 755 (Montgomery and colleagues, 1962). The activity of the nitrosoureas against experimental animal neoplasms (Skiper and coworkers, 1961) led to the obtention of cytostatic purines with side chains containing a nitrosoureido function (Johnston and colleagues, 1963) and prompted to synthesize various nucleosides with this alkylating function attached to the carbohydrate moiety (Lin and colleagues, 1978; Montgomery and Thomas, 1979). Recently, the cytotoxic action of certain triazene derivatives (Audette and coworkers, 1973; Connors and coworkers, 1976), such as 4-(3,3-dimethyl-1-triazeno)imidazole-5-carboxamide (DTIC), effective in the treatment of melanoma (Kleihues and colleagues, 1976) has led to the preparation of a riboside derivative, namely 4-(3,3-dimethyl-1-triazeno)-1-(β-D-ribofuranosyl) imidazole-5-carboxamide which displays antileukemic activity (Panzica and Townsend, 1971); Earl and Townsend, 1979).

As shown, β-chloroethylamines, β-chloroethylsulphides, aziridines, nitrosoureas and triazenes have been used in the preparation of alkylating nucleic acid components and related compounds. Other functional groups with alkylating ability, such as epoxides and alkanesulfonates, have also been utilized as cytotoxic groupings of alkylating agents (Sartorelli and Johns, 1975). There are, however, some other chemically efficient alkylating groups, such as allylic or bencylic type halides, that have not been used as the active moieties of potential antitumor drugs. With the aim of examining the possibilities of the benzylic type halides as a new type of cytotoxic alkylating agents, we have studied the synthesis, cytostatic activity and mode of action of a series of N-glycosyl pentaheterocyclic compounds (1) in which the halomethyl group, as alkylating moiety, is attached to the heteroaromatic ring.



1, X = halogen
Gl = glycosyl

In order to study the influence of the carrying structure on cytostatic activity, alkylating 1,2,3-triazoles, pyrazoles, imidazoles and 1,2,4-triazoles, with different substituents on the ring and on N-1 were prepared.

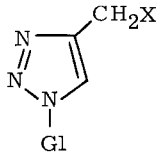
CHEMISTRY

1,2,3-Triazole Nucleosides

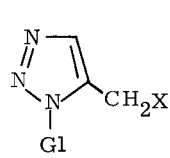
Alkylating 1,2,3-triazole nucleosides have been obtained by 1,3-dipolar cycloaddition of glycosyl azides to halomethyl acetylenes. A number of glycosyl moieties having different size, stereochemistry, type of sugar ring and protecting groups for the sugar hydroxyls, and a non glycosylic moiety have been employed[1] (De las Heras and coworkers, 1979, 1980a; Camarasa and coworkers, 1980; Alonso and coworkers, 1980). Thus, reaction of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl azide (2a), 2,3,5-tri-O-acetyl-β-D-ribofuranosyl azide (2b), 2,3,4-tri-O-acetyl-β-D-ribopyranosyl azide (2c), 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl azide (2d) or 2,3-O-isopropylidene-5-O-trityl-α-D-ribofuranosyl azide (2e), with unsymmetric dipolarophyles, propargyl chloride or propargyl bromide, gave a mixture of the two possible 4-chloromethyl- and 5-chloromethyl-1-glycosyl-1,2,3-triazole isomers 4 and 8 or 4-bromomethyl- and 5-bromomethyl-1-glycosyl-1,2,3-triazoles 5 and 9, respectively, in which the sterically less hindered 4-halomethyl derivatives 4 and 5 were the major products. However, reaction of 2,3,5-tri-O-benzoyl-β-D-ribofuranosyl azide (2f), 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl azide (2g), 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl azide (2h) or the non glycosylic benzyl azide (2i) with the same unsymmetric propargyl halides gave the less hindered isomers 4 and 5 as the only product. Cycloaddition reaction of glycosyl azides 2a, 2b, 2f, 2g and 2h with symmetric dipolarophyle 1,4-dichlorobutyne, or reaction of glucopyranosyl azide 2a with 1,4-dibromobutyne afforded 1-glycosyl-4,5-bis(chloromethyl)1,2,3-triazoles 11 or 1-glucopyranosyl-4,5-bis(bromomethyl)-1,2,3-triazole 12a. As expected, both conformation of the sugar moiety and anomeric configuration of the halomethyl-1,2,3-triazole nucleosides were identical to those of the starting azide.

A second route has been employed to prepare iodomethyl- and some bromomethyl-1,2,3-triazole alkylating nucleosides, which could not be obtained by the above mentioned method. It has also been employed to synthesize some of the chloromethyl- and bromomethyl-1,2,3-triazole glycosides above described, although lower yields were obtained. This second route consisted on the 1,3-dipolar cycloaddition of glycosyl azides to hydroxymethyl acetylenes to afford 1-glycosyl-hydroxymethyl-1,2,3-triazoles, followed by substitution of the hydroxy group by a halogen atom. Thus, reaction of glycosyl azides 2a, 2c, 2d and 2i with propargyl alcohol gave a mixture of the two possible 1-glycosyl-4-hydroxymethyl-7, and 1-glycosyl-5-hydroxymethyl-1,2,3-triazole 10. Similarly, reaction of glycosyl azides 2a, 2b and 2d with 1,4-dihydroxybutyne afforded 1-glycosyl-4,5-bis(hydroxymethyl)-1,2,3-triazoles 14. Transformation of hydroxymethyl-1,2,3-

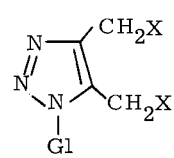
[1] Throughout this paper the small letter identifies always the same glycosyl moiety.



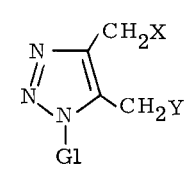
3, X = F
4, X = Cl
5, X = Br
6, X = I
7, X = OH



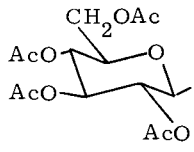
8, X = Cl
9, X = Br
10, X = OH

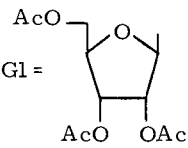


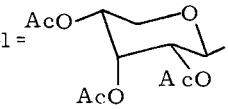
11, X = Cl
12, X = Br
13, X = I
14, X = OH

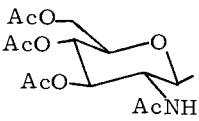


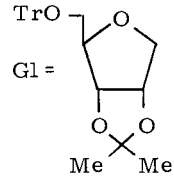
15, X = Cl; Y = I
16, X = I ; Y = Cl

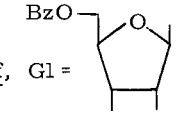
a, Gl = 

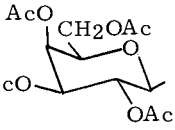
b, Gl = 

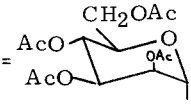
c, Gl = 

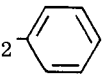
d, Gl = 

e, Gl = 

f, Gl = 

g, Gl = 

h, Gl = 

i, Gl = 

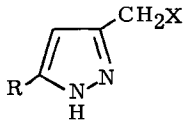
triazole derivatives 7i and 14a to the corresponding chloromethyl analogs 4i and 11a, respectively, was carried out by reaction with triphenylphosphine/carbon tetrachloride in acetonitrile. Synthesis of bromomethyl-1,2,3-triazole derivatives 5i, 12a and 12d was accomplished by treatment of the hydroxymethyl compounds 7i, 14a and 14d, respectively, with triphenylphosphite/bromine in 1,2-dimethoxyethane. Similarly, reaction of 1-glycosyl-hydroxymethyl-1,2,3-triazoles 7a, 7c and 7i with thriphenylphosphite/iodine in 1,2-dimethoxyethane afforded iodomethyl derivatives 6a, 6c and 6i, respectively. All these halogenation reactions gave good yields, however the overall yield was not high because of the usually low yields obtained from the cycloaddition reactions of hydroxymethyl acetylenes.

Alkylating nucleosides have also been obtained by interchange of halogen from a halomethyl-1,2,3-triazole derivative synthesized by the above methods. Thus, 1-benzyl-4-fluoromethyl-1,2,3-triazole (3i) was prepared by mild treatment of the bromomethyl analog 5i with anhydrous potassium fluoride in the presence of 18-crown-6. 4-Iodomethyl derivatives 6b, 6e, 6g and 6h were easily obtained by transhalogenation reaction of the corresponding 4-chloromethyl- and/or 4-bromomethyl-1,2,3-triazole analogs 4 and/or 5, respectively, with sodium iodide. In the case of 4,5-bis(chloromethyl) derivatives, such as 11g and 11h, treatment with one equivalent of sodium iodide afforded mixtures of the two possible isomers 4-chloromethyl-5-iodomethyl-1,2,3-triazole, 15, and 5-chloromethyl-4-iodomethyl-1,2,3-triazole 16. However, reaction of 11g with an excess of iodinating reagent gave the 4,5-bis(iodomethyl)-1,2,3-triazole 13g.

Pyrazole Nucleosides

Halomethylpyrazole nucleosides were prepared by two different ways involving glycosylation of the corresponding halomethyl pyrazolic bases or generation of the alkylating halomethyl group on suitable substituted pyrazole nucleosides previously formed (García-López and colleagues, 1979, 1980a, 1980b).

Chloromethyl- and bromomethylpyrazoles 17, 18 and 19, 20 were prepared by treating the corresponding hydroxymethyl derivative with thyonyl chloride and phosphorus tribromide respectively. Reaction of 17 and 18 with sodium iodide provided the iodomethylpyrazoles 21 and 22.



17, X = Cl; R = CO_2Et 20, X = Br; R = $CONH_2$

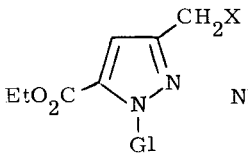
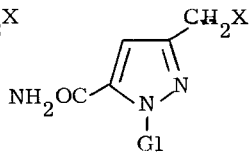
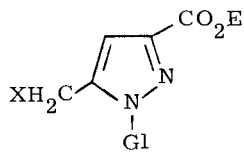
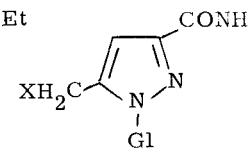
18, X = Cl; R = $CONH_2$ 21, X = I ; R = CO_2Et

19, X = Br; R = CO_2Et 22, X = I ; R = $CONH_2$

Glycosylation of all these halomethylpyrazoles 17-22 with penta-O-acetyl-β-D-glucopyranose and tetra-O-acetyl-β-D-ribofuranose, or reaction of 19 and 20 with tetra-O-acetyl-β-D-ribopyranose, by the fusion method (Watanabe and colleagues, 1974), using p-toluenesulfonic acid as catalyst, gave the 3-halomethyl substituted nucleosides 23-25, 27-29 or a mixture of these 3-halomethyl derivatives and their 5-halomethyl substituted isomers 31-34 respectively, all of them with a β configuration. From the reaction of 17-19 with penta-O-acetyl-β-D-glucopyranose, small amounts of the 3-halomethyl substituted nucleosides with α configuration were also obtained (23aα, 27aα and 24aα, respectively).

Only peracetylated sugars were used as the glycosyl moiety of these pyrazole nucleosides, since it was previously demonstrated in the series of 1,2,3-triazole alkylating nucleosides that one of the most important factors in conditioning cytostatic activity is the sugar hydroxyl protecting groups, being acetyl group the more suitable of those checked (Camarasa and colleagues, 1980).

The mentioned glycosylation procedure gave good total yields of nucleoside products when the starting bases were 17-20. However, when the iodomethyl

23, X = Cl
24, X = Br
25, X = I
26, X = $(CH_2CH_2Cl)_2$

27, X = Cl
28, X = Br
29, X = I
30, X = $N(CH_2CH_2Cl)_2$

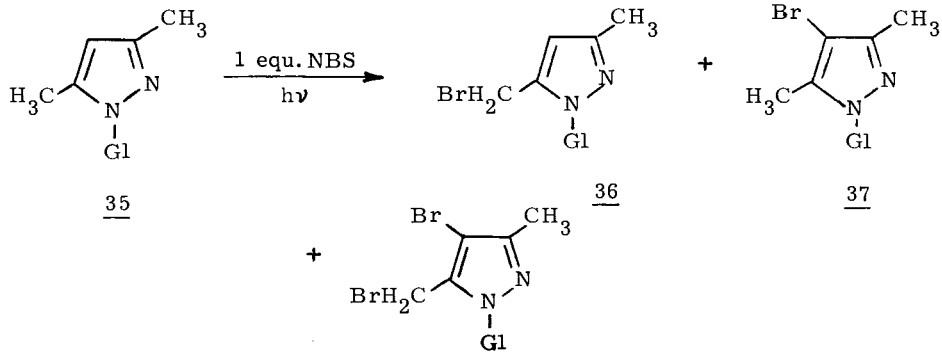
31, X = Br

32, X = Cl
33, X = Br
34, X = I

derivatives 21 and 22 were used, very poor yields of nucleosidic material were obtained. Iodomethyl substituted nucleosides 25, 29 and 34 were prepared in high yields from the chloromethyl nucleoside analogs by chlorine-iodine exchange.

On the other hand, pyrazole nitrogen mustard nucleosides 26 and 30 were obtained by treatment of the corresponding 3-bromomethyl substituted analogs 24 and 28 with bis(2-chloroethyl)amine. These nitrogen mustard derivatives were prepared to compare the relative cytostatic effect that these two different alkylating groups (halomethyl and bis(2-chloroethyl)aminomethyl) have on the same carrier.

The difficulty in obtaining 3(5)-methyl-5(3)-(bromomethyl)pyrazole, as well as other N-unsubstituted bromomethylpyrazoles without withdrawing substituents, for its subsequent glycosylation, led us to undertake a different route for the synthesis of nucleosides of such pyrazole. This alternative route consisted on the generation of the alkylating bromomethyl group from the reaction of 3,5-dimethylpyrazole nucleosides, 35, previously formed, with N-bromosuccinimide (NBS).



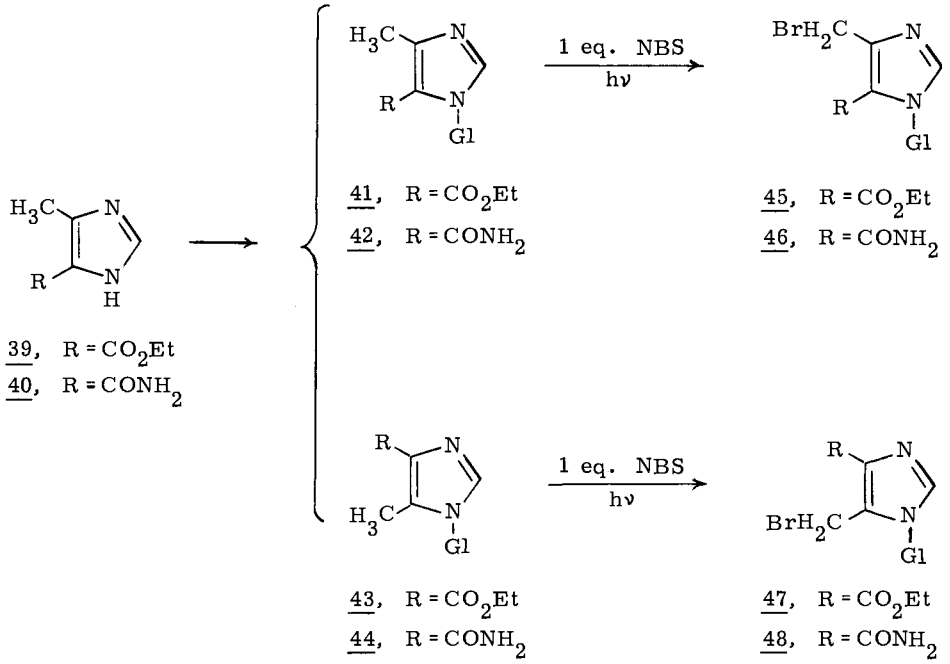
35 → (1 equ. NBS, hν) → 36 + 37 + 38

As previously described for 1-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-3,5-dimethylpyrazole (35a)(Jasinska and Sokolowski, 1970), the 1-β-D-ribofuranosyl derivative, 35b, was obtained by glycosylation of 3,5-dimethylpyrazole via mercuric cyanide-nitromethane method (Yamaoka and coworkers, 1965).

Bromination of 35a and 35b with one equivalent of NBS in carbon tetrachloride and irradiating with a 200 watt lamp gave a mixture of 1-glycosyl-3-methyl-5-(bromomethyl) pyrazole (36) and 1-glycosyl-4-bromo-3,5-dimethylpyrazole (37) along with minor amounts of the dibrominated compound 1-glycosyl-3-methyl-4-bromo-5-(bromomethyl) pyrazole (38). Although the total yield of bromination for 35a and 35b was similar, the ratio of monobrominated compounds on the side chain or on the pyrazole ring varied with the starting material. Thus, in the case of the 1-glucosyl derivative, 35a, the desired 5-bromomethyl substituted compound 36a predominated. However, the 1-ribosyl derivative, 35b, gave the 4-bromo substituted compound 37b as the major product. Bromination of 35a and 35b with two equivalents of NBS under the same conditions as above gave essentially the dibrominated compounds 38a and 38b, respectively, along with very minor amounts of 36 and 37. Attempts to introduce a second bromomethyl alkylating group by adding larger amounts of NBS furnished intractable mixtures of compounds.

Imidazole Nucleosides

The synthesis of nucleosides of 4(5)-(bromomethyl) imidazole-5(4)carboxylate and 4(5)-(bromomethyl) imidazole-5(4)-carboxamide was achieved by bromination of the corresponding methyl substituted nucleosides with NBS, following the same route as that employed for the preparation of the bromomethylpyrazoles 36 and 38 above described (García-López and Herranz, 1980c).



39, R = CO_2Et
40, R = $CONH_2$

41, R = CO_2Et
42, R = $CONH_2$

45, R = CO_2Et
46, R = $CONH_2$

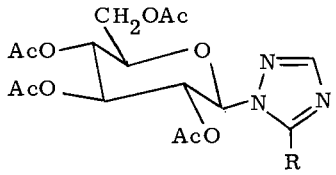
43, R = CO_2Et
44, R = $CONH_2$

47, R = CO_2Et
48, R = $CONH_2$

Methylimidazole nucleosides 41–44 were obtained by glycosylation of the corresponding bases 39 and 40 with acetylated glucopyranosyl and ribofuranosyl halides via mercuric cyanide–nitromethane method. In all cases, the two possible positional isomers were obtained, exception being reaction of 40 with 2,3,5-tri-O-acetyl-D-ribofuranosyl chloride, in which the 5-methyl-4-carboxamide substituted nucleoside 44b was the only reaction product. Bromination of all these methylimidazole nucleosides with one equivalent of NBS in carbon tetrachloride or ethyl acetate, irradiating with a 200 watt lamp, gave, in each case, the corresponding bromomethylimidazole carboxylates, 45 and 47, or carboxamides 46 and 48 as the only reaction products. In contrast to the case of methylpyrazole nucleosides above described, substitution on the heteroaromatic ring was not observed. Bromomethylimidazole nucleosides were, in general, unstable compounds which decomposed on standing. Due to this instability, the more chemically alkylating iodomethyl substituted derivatives were not prepared.

1,2,4-Triazole Nucleosides

The starting compound, 1-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-1,2,4-triazole (49), for the preparation of 1,2,4-triazole alkylating glucosides (De las Heras and Camarasa, 1980b) was obtained by glucosylation of 1,2,4-triazole according to the mercuric cyanide-nitromethane method. Glucosylation by other methods, such as the fusion method or the trimethylsilyl derivative method, gave lower yields and several byproducts. Hydroxymethylation of 49 with aqueous formaldehyde gave the 5-hydroxymethyl-1,2,4-triazole glucoside 50, which was converted to the halomethyl-1,2,4-triazole derivatives using the triphenylphosphine and triphenylphosphite halogenating reagents already mentioned under 1,2,3-triazoles. Treatment of 50 with triphenylphosphine/carbon tetrachloride



49, R = H
50, R = CH_2OH
51, R = CH_2Cl
52, R = CH_2Br
53, R = CH_2I
54, R = CH_2-N⟨ ⟩
55, R = $CH_2N(CH_2CH_2Cl)_2$

gave a good yield of the 5-chloromethyl-1,2,4-triazole 51. Similarly, reaction of 50 with triphenylphosphite/bromine afforded the 5-bromomethyl compound 52. However, treatment of 50 with triphenylphosphite/iodine, which should have given the iodomethyl compound 53, afforded the starting compound 50 unchanged. The 5-iodomethyl-1,2,4-triazole derivative 53 was obtained in good yield by transhalogenation reaction of the 5-chloromethyl-1,2,4-triazole glucoside 51 with sodium iodide.

Other "classical" alkylating groups were also incorporated to this glucosyl-1,2,4-triazole residue in order to compare their cytostatic activity with that of the

benzylic type halomethyl-1,2,4-triazoles. Thus, reaction of 5-chloromethyl-1,2,4-triazole 51 with aziridine gave the 5-(N-aziridinomethyl)-1,2,4-triazole glucoside 54, and treatment of the 5-bromomethyl analog 52 with bis-(2-chloroethyl) amine afforded the nitrogen mustard compound 55.

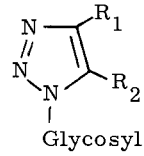
Several attempts of acid hydrolisis were made to obtain deprotected alkylating nucleosides, in order to compare their cytostatic activities with those of acetyl, benzoyl and ether protected nucleosides. Thus, the obtention of 4-iodomethyl-1-(α-D-ribofuranosyl)-1,2,3-triazole was attempted by treatment of the corresponding 2,3-O-isopropylidene-5-O-trityl derivative 6e with an ethanolic anhydrous solution of HCl. Similarly, the preparation of 4,5-bis-(chloromethyl)-1-(β-D-glucopyranosyl)-1,2,3-triazole from the corresponding peracetylated derivative 11a was also attempted by treatment of the latter with anhydrous zinc chloride. These attempts led to unstable syrups, which after several chromatographic purifications could not be obtained analytically pure. Basic hydrolysis (methanolic ammonia) gave, as expected, not only deacetylation but also the substitution of the halo atom to yield the corresponding aminomethyl derivatives.

CYTOSTATIC ACTIVITY

The in vitro cytostatic activities of all these alkylating derivatives were evaluated against HeLa cells following described methods (Geran and colleagues, 1972). The more active compounds in this preliminary test were assayed for their cytostatic activities in mice bearing tumors according to protocols of NCI (Geran and colleagues, 1972).

Two structural aspects essentially determined the cytostatic activity of these series of 1,2,3-triazole, pyrazole, imidazole and 1,2,4-triazole alkylating derivatives, namely the influence of the N-1 substituent and the influence of the alkylating ability of the $-CH_2X$ halomethyl group. According to the first aspect, only the use of some peracetylated glycosyl moieties gave compounds with significant activities. Thus, N-1 unsubstituted halomethylpyrazoles 17-22 were completely devoid of in vitro cytostatic activity ($ED_{50} > 100$ μg/ml), while non-glycosidic 1-benzyl-4-(halomethyl)-1,2,3-triazoles 4i-6i showed in vitro activity but did not increase the life span of mice bearing ECA tumor. Likewise, none of the alkylating nucleosides bearing tetraacetyl-β-D-glucosamine (4d, 5d and 8d), tribenzoyl-β-D-ribofuranosyl (4f, 5f and 11f) and isopropylidene trityl-α-D-ribofuranosyl 4e-6e and 8e) gave significant cytotoxicities. The lack of activity of alkylating nucleosides bearing the two latter glycosyl moieties may be explained on the basis of transport difficulties, while the low activity of the tetraacetyl-β-D-glucosamine derivatives may be due to instability as will be discussed later. The only active compounds in mice bearing tumors were the (bromomethyl)- and (iodomethyl) azole derivatives having tetraacetyl-β-D-glucopyranosyl (5a, 6a, 28a and 29a), triacetyl-β-D-ribofuranosyl (29b, 33b and 34b), tetraacetyl-β-D-galactopyranosyl (5g, 6g and 15g+16g) and tetraacetyl-α-D-mannopyranosyl (5h) moieties (see Tables 1 and 2). These facts indicate that the presence of acetyl protected glycosyl moieties is required for cytostatic activity in these compounds. The nature of the sugar seems not to be determinant of cytostatic activity, since the four sugar moieties used i.e., glucosyl, ribosyl, galactosyl

TABLE 1 Cytostatic Activities of 1-Glycosyl-halomethyl-1,2,3-triazoles

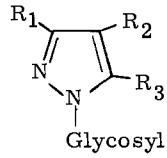


Compd[a]	R_1	R_2	HeLa cells ED_{50} μg/mL	Tumor[b]	Optimal dosage mg/Kg[c]	T/C x 100[d]
5a	CH_2Br	H	3.5	B1	100	134
5a	CH_2Br	H		ECA	75	145
5a	CH_2Br	H		LE		-
5a	CH_2Br	H		PS	50	163
5b	CH_2Br	H	5			
5c	CH_2Br	H	2.5	ECA		-
5g	CH_2Br	H	9	ECA	75	158
5h	CH_2Br	H	6	ECA	100	150
5i	CH_2Br	H	2	ECA		-
6a	CH_2I	H	2	ECA	100	195
6a	CH_2I	H		PS		-
6b	CH_2I	H	3.5			
6c	CH_2I	H	1.5	ECA		-
6g	CH_2I	H	5	ECA	25	133
6h	CH_2I	H	5	ECA		-
6i	CH_2I	H	2	ECA		-
9a	H	CH_2Br	4	ECA		-
11a	CH_2Cl	CH_2Cl	6			
11b	CH_2Cl	CH_2Cl	2.5			
11g	CH_2Cl	CH_2Cl	4	ECA	100	141
11h	CH_2Cl	CH_2Cl	5			
12a	CH_2Br	CH_2Br	3	ECA		-
13g	CH_2I	CH_2I	0.7	ECA		-
15g & 16g	CH_2Cl-CH_2I		0.9	ECA	25	133
15h & 16h	CH_2Cl-CH_2I		0.8			

[a] The small letter following the number refers to the N-1 substituent according to the meanings indicated under Chemistry of 1,2,3-triazoles. [b] B1 = B16 Melanocarcinoma; ECA = Ehrlich Carcinoma Ascites; LE = L-1210 Lymphoid Leukemia; PS = P-388 Lymphocytic Leukemia. [c] Administered once daily for 9 consecutive days beginning 24 h after tumor implantation. [d] T/C is the ratio (expressed as a percentage) of the median survival time of the treated group of mice divided by the median survival time of the control group. A value of T/C x 100 $\geq$ 125 is considered a statistically significant indication of antitumor activity. A stroke in this column means that the determined T/C x 100 value was lower than 125.

and mannosyl, when acetylated and attached to a suitable halomethyl azole, gave compounds which increased the life span of mice bearing tumors. Similarly, the type of the ring (pyranose or furanose), the size (pentose or hexose) or the α or β anomeric configuration of the sugar were not conclusive, since the four mentioned glycosyl moieties covered all these structural possibilities and all of them gave compounds with similar cytostatic activities.

TABLE 2 Cytostatic Activities of 1-Glycosyl-halomethyl-pyrazoles



Compd[a]	R_1	R_2	R_3	HeLa cells ED_{50} μg/mL	In mice bearing ECA tumor[b] Optimal dosage mg/Kg[c]	T/C x 100[d]
24a	CH_2Br	H	COOEt	5		-
24a	CH_2Br	H	COOEt	5		
24b	CH_2Br	H	COOEt	2.5		-
24c	CH_2Br	H	COOEt	2		
25a	CH_2I	H	COOEt	2.5		
25b	CH_2I	H	COOEt	5.5		
28a	CH_2Br	H	$CONH_2$	4	12.5	176
28b	CH_2Br	H	$CONH_2$	2		
28c	CH_2Br	H	$CONH_2$	2		
29a	CH_2I	H	$CONH_2$	2	50	176
29b	CH_2I	H	$CONH_2$	1.5	25	158
31a	COOEt	H	CH_2Br	6		-
33b	$CONH_2$	H	CH_2Br	2	12.5	134
34b	$CONH_2$	H	CH_2I	2	25	141
36a	CH_3	H	CH_2Br	4		-
36b	CH_3	H	CH_2Br	13		
38a	CH_3	Br	CH_2Br	8		
38b	CH_3	Br	CH_2Br	7		

a, b, c, d See notes of Table 1.

The second common aspect in the SAR of these compounds is that cytostatic

activity within a series increased with the alkylating ability of the $-CH_2X$ halomethyl group. According to this, no activity was found either for the non alkylating hydroxymethyl derivatives 7, 10, 14 and 50 or fluoromethyl derivative 3i. Cytotoxicity of mono(chloromethyl) derivatives was higher. However, they never reached significant values. Thus, cytotoxicities of the 4- and 5-(chloromethyl)-1,2,3-triazoles 4a-i and 8a-e, 3- and 5-(chloromethyl)pyrazoles 23a,b, 27a,b and 32, and 5-(chloromethyl)-1,2,4-triazole 51 were not significant (ED_{50} values, not shown in Tables were between 10 and 100 μg/ml). However, the corresponding more alkylating 4- and 5-(bromomethyl)-1,2,3-triazoles 5a-c, 5g-i and 9a, 4-(iodomethyl-1,2,3-triazoles 6a-c, 6g-i, 3- and 5-(bromomethyl) pyrazoles 24a,b, 28a,b and 33b, 3- and 5-(iodomethyl) pyrazoles 25a,b, 29a,b and 34b, and 5-(bromomethyl)- and 5-(iodomethyl)-1,2,4-triazoles 52 and 53[2], all of them with suitable glycosyl moieties, gave significant in vivo and/or in vitro cytostatic activities (Tables 1 and 2). The 4-(bromomethyl)- and 4-(iodomethyl) -1,2,3-triazoles 5d-f and 6e,f with unsuitable glycosyl moieties were not significant cytostatics.

The substitution position of the halomethyl group was not important, since isomeric 4-(halomethyl) and 5-(halomethyl)-1,2,3-triazoles or 3-(halomethyl) and 5-(halomethyl) pyrazoles gave similar cytostatic activities.

Although, on the whole, the activities against HeLa cells of the 3-methyl substituted pyrazoles 36a,b and 38a,b were a little lower than those of the carboxylate and carboxamide substituted analogs 24a,b, 28a,b, 31a and 33b, the nature of the substituent, different from the alkylating halomethyl group, did not generally affect the in vitro cytostatic activity. However, in the in vivo assays in mice bearing ECA tumor, only those carboxamide substituted (bromomethyl)- and (iodomethyl)pyrazoles 28a, 33b and 29a,b, 34b, respectively, were found to be active (compounds 28b,c have not been tested).

In spite of possessing the two structural requirements, halomethyl group with high alkylating ability, and "suitable" glycosyl moiety, none of the peracetylated glucopyranosyl- and ribofuranosyl-(bromomethyl)imidazole derivatives 45-48 showed significant cytostatic activity (ED_{50} not shown in Tables were between 10 and 100 μg/ml). This lack of activity is probably due to instability, since, as already said, all of these bromomethylimidazole nucleosides easily decomposed on standing. The ineffectiveness as cytostatics of unstable alkylating halomethyl azole nucleosides is a common fact which has been observed in several cases. Thus, the above mentioned low activity of the tetraacetyl-β-D-glucosamine derivatives 4d, 5d and 8d, as compared with that of the corresponding tetraacetyl-β-D-glucose derivatives 4a, 5a and 8a could be explained on the basis of instability of the halomethyl group which could react with the C-2' acetamide AcNH group of the sugar residue. Moreover, although 4-(bromomethyl)- and 4-(iodomethyl)-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1,2,3-triazole (5b and 6b) showed significant in vitro activity (Table 1), because of their instability they were not tested in mice bearing tumors.

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[2] Cytostatic activities against HeLa cells of halomethyl-1,2,4-triazoles, 51, 52 and 53, expressed as ED_{50}, were 25 and 2.5 μg/ml, respectively.

The presence of a second alkylating group in the difunctional 4,5-bis(halomethyl)-1,2,3-triazoles gave more active and more toxic compounds than the corresponding monofunctional derivatives. Thus, peracetylated glucosyl derivatives of 4,5-bis(chloromethyl)-1,2,3-triazole 11a,b,g,h were active in the in vitro test and 11g also in mice bearing ECA tumor. Similarly, 4,5-bis (bromomethyl) 12a, 4,5-bis (iodomethyl), 13g,h and the pair of isomers 4-(chloromethyl)-5-(iodomethyl) and 4-(iodomethyl)-5-(chlromethyl)-1,2,3-triazoles 15g,h and 16g,h gave the highest cytotoxicities against HeLa cells but were very toxic to mice bearing ECA tumor. Only when administered at low doses (25 mg/Kg) higher T/C values were obtained, which in no case, exception being 15g and 16g, reached the significant value of T/C x 100 $\geqslant$ 125.

Finally, substitution of the effective bromomethyl or iodomethyl groups in the pyrazole glycosides 24a-c, 28a-c or 25a,b, 29a,b and in the 1,2,4-triazole glucosides 52 or 53 by the "classical" alkylating groups, nitrogen mustard and/or aziridine gave the non active pyrazole nitrogen mustards 26-a-c and 30a-c, 1,2,4-triazole nitrogen mustard 55 and 1,2,4-triazole aziridine 54.

MODE OF ACTION

The mode of action of some of the 1,2,3-triazole and pyrazole derivatives shown in Tables 1 and 2 have been studied (Contreras and coworkers, 1978). The same type of experiments were carried out for both series and similar results were obtained. The following discussion refers to 1,2,3-triazole but can be extended to pyrazole derivatives. As preliminary experiments the effects of halomethyl azole derivatives on the synthesis of macromolecules (nucleic acids and proteins) were studied. 4-Iodomethyl-1-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-1,2,3-triazole (6a) inhibited DNA synthesis by ECA cells, as measured by (methyl-3H)thymidine incorporation. This inhibition was complete and irreversible. This fact may imply that DNA is damaged (alkylated) by these halomethyl derivatives (Painter, 1977). The synthesis of RNA and proteins, as determined by incorporation by ECA cells of (5,6-^{3}H)uridine and (5-^{3}H)proline, respectively, was inhibited to a lesser extent.

The structure of these compounds, having a benzylic type halomethyl group, and their preferential (Wheeler, 1962; Sartorelli and Johns, 1975) and irreversible (Painter, 1977) inhibition of DNA synthesis, suggested that they act as alkylating agents. To confirm this suggestion, the incorporation of tritiated methyl-groups from di(^{3}H) methylsulfate by ECA cells in the presence of 4-bromomethyl-1-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-1,2,3-triazole (5a) and the above mentioned 6a was studied. These two compounds inhibited the incorporation of radioactivity from this radioactive, typically alkylating agent which preferentially alkylates N-7 position of guanine moieties (Lawley and Brookes, 1964; Wheeler, 1962). By this reason the method of Tomasz (1970) was followed to detect alkylation at that position of guanine, by the concurrent labilization and release of ^{3}H attached to C-8. This experiments showed that N-7 is the main site of alkylation. According to this method ECA cells were prelabeled in the presence of (methyl-^{3}H)thymidine or (8-^{3}H) guanosine, and then incubated in the presence of compound (6a). The amount of radioactivity remaining in the nucleic acids of the prelabeled cells did not decreased when DNA was pretreated with

(8-^{3}H) guanosine, the loss of radioactivity was about 70%. This result was in agreement with the known ability of alkylating agents of mainly binding to N-7 of guanine, promoting both the labilization of ^{3}H attached to C-8 of guanine and the cleavage of the C-1'-N-9 nucleosidic bond with subsequent release of the alkylated base (Lawley, 1967; Sartorelli and Johns, 1975; Wheeler, 1962).

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We dedicate this paper to the memory of our dear Master, Professor Guillermo García-Muñoz, who died on March 1976.

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Molecular Engineering: A General Approach to QSAR

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ABSTRACT

A short review of QSAR Techniques is the starting point to collect a wider class of mathematical and physical tools. The individuals of a molecular family are points in the n-dimensional space of molecular properties and theoretical parameters. The important fact of this space's disconnection is discussed and a general framework to find the possible links between molecular properties and theoretical parameters is shown. The parameters' role as building blocks in QSAR is considered in a general scope, from the more classical ones, as Hansch's π to the Quantum Mechanical, as density functions. The widening of mathematical tools and theoretical parameters suggest that out of QSAR it may emerge a more general art: Molecular Engineering. Two examples are given. First, phenols are treated with principal components method and Quantum Mechanical parameters. The resulting multivariate correlations are compared with previous calculations and the possibility of quadratic terms in order to optimize the molecular design is analyzed. Second, pattern recognition ideas and density functions are used to obtain an order relation in the hexane isomers family. The results are encouraging and give much better descriptive relationships than previous studies.

KEYWORDS

Quantitative Structure - Activity Relationships; Molecular Engineering; Multivariate Analysis; Pattern Recognition; Density Functions.

INTRODUCTION

Quantitative Structure - Activity Relationships (QSAR) is

not a modern Science topic, but an old dream of mankind. The numerology related to the use and administration of drugs is an ancestrial craft of man, secret and ersteric and sometimes a very successful one. As in the case of Arnau de Vilanova (1975) catalan, heterodox, philosoper and physician - protected by popes and kings - who in a medieval booklet speculates on the right proportions of the elementary properties of matter - air, fire, water and earth - which should be contained in a given drug or prescription. But laying aside Alchemy, it seems that the real starting point of QSAR may be associated to the ideas of Overton (1897) and Meyer (1899) - relating empirical parameters to biological activity - on which is based the main body of relations known today as Hansch analysis (1969). Other early attempts make connections between quantum chemical parameters and physical properties (Del Re, 1958; Streitweser, 1961). In later works, the parameters to be related with biological or physicochemical properties are obtained by means ranging from quantum mechanical calculations (Purcell et. al, 1973) to Graph theory (Randic, 1978).

It is out of reach from a limited paper like this to provide an exhaustive, and perhaps boring, review of the whole existent informative material, but a good example of the volume covering the possible quotations on the current QSAR work may be obtained by inspection of the following Table 1, which shows the evolution of the amount of literature in the late 1970s. There is apparent an explosive growth of the number of papers, searched under the keyword QSAR, which starts to show up around 1975. If the trend is maintained through the next decade, by 1990 one can expect, using an exponential fit, around 7000 papers on the subject!

Table 1. QSAR quotation index

Year	Number of articles*
1969	1
70	2
71	5
72	8
73	7
74	10
75	20
76	30
77	41
78	31
79	104

*Source: Chemical Abstracts

At the view of these figures it seems useless to insist on a comprehensive review, on the contrary the situation looks like asking for a synthetic attempt, and the present study will try to start such a way, looking ahead for a more detailed account which may be published elsewhere.

First, we must try a redefinition of the application fields of QSAR and a widening of the underlying mathematical philosophy by considering the n-dimensional molecular spaces of molecular properties and parameters. At this stage, the capital role of such

parameters will be evident and a classification based on their origin and definition may consitute a further help to understand the actual state and the future possibilities of the QSAR. Next, the methodological tools developed in order to extract hidden information from biological data and experimental molecular parameters must be described in the widest possible way, followed by auxillary and parallel techniques, which may be worth being used, when the QSAR goal is stated as to obtain, by the shortest path, a taylor made structure to fit a given objective. As we said, this ambitious planning should be moderated by the lack of space. Our aim here is to present more than concrete results, the basis of a general and open framework in order to amalgam many ideas developed in somewhat independent ways, to reconcile apparently incompatible points of view, and, finally, from a pragmatic position, to show that many paths can be followed in order to obtain a desired optimal molecular design. Finally, we think that this widening of QSAR ideas deserves a more general name and we support the suggestion of calling it Molecular Engineering.

MOLECULAR SPACES

In the physical and chemical description of molecular structure there is the implicit assumption that a molecule, as an individual stable object, belongs to some n-dimensional space. When molecular parameters are listed in some ordered manner for a given chemical species, when chemists draw a bond structure or theoreticians compute molecular wavefunctions, there is always present a one-to-one correspondence between the molecular image and a vector belonging to some n-dimensional space. The nature of this molecular space depends on the image chosen to represent each molecule.

In these spaces a metric can be defined, some axiomatic rule which transforms pairs of molecular points into real numbers. Then, distance relationships and other mathematically similar devices may be used. In fact, the metric possibility constitutes a fundamental relationship upon which the QSAR mainframe is based.

Trying to synthesize and to enlarge QSAR ideas, let's suppose that a part, M_k, of a molecular family, M, is known; two well defined sets sets may be attached to M_k: the set Π_k of chemical, physical and biological properties containing information gathered, say, experimentally and the set P_k of parameters, usually obtained from theoretical sources, containing usual information on M_k. The information on both sets can be expressed in matrix form: to Π_k we can attach the matrix $\underline{\Pi}_k$ and to P_k the matrix $\underline{P}_k$. Each row of these matrices - one vector in the n-dimensional space of properties and parameters - is related to each element of M_k and each column to a property or parameter. Suppose that our primary aim is to obtain, if it exists, some sort of relationship between the sets Π_k and P_k as a predicitve tool for the unknown part, M_u, of the family M. Then, the problem is to structure the kind of relationship we are looking for. A general and commonly used form may be summarized in a set of multivariate functions $\Phi = \{\varphi_j\}$, relating each property, Π_{kj}, in Π_k to the set of parameters in P_k. That is

$$\Pi_{kj} = \varphi_j (P_k) \qquad (1)$$

The function φ_j in (1) may in turn be expressed as a power series of the parameter set $P_k = \{p_i\}$:

$$\varphi_j(P_k) = a_o^j + \sum_{\ell} a_{s,\ell}^j p_\ell + \sum_{\ell}\sum_{m} a_{2,\ell m}^j p_\ell p_m + \ldots \qquad (2)$$

So, least squares techniques may eventually be used to fit the coefficients $\{a_s^j, a_{s,\ell}^j \ldots\}$ to the values of the matrix $\underline{\Pi}_k$. This is achieved by minimizing the norm:

$$\varepsilon_2 = |\underline{\Pi}_k - \underline{Z}|^2 \qquad (3)$$

where the matrix Z is constricted with the values of the parameter matrix $\underline{P}_k$, whose rows are $p_{i\cdot}$, and the function set Φ.

$$Z_{ij} = \varphi_j(p_{i\cdot}).$$

The minimum value of ε_2 is related to the fitness of the set Φ to reproduce the properties Π_k.

When the functions φ_j are applied to $\underline{P}_u$, then, they can reproduce the properties Π_u of the unknown part $\underline{M}_u$ of the family M. Usual practice constraints Π and Φ to one element, and only linear terms are used in the expansion (2). But, as we have seen, nothing is against to construct a completely general framework, given a molecular family. If the set Φ is obtained, subsequent manipulation of the available data is possible. For example, one can try to obtain optimal points on the function set Φ, or what is more tempting, use it to test theoretically the adequate structure of new molecules. From here a very promising collection of pathways starts.

As an example, which seems absent from the current literature as far as we know, let's suppose that two properties are determined on M_k, thus $\Pi = \{\Pi_1, \Pi_2\}$. It is not out of the current situations that, say, Π_1 corresponds to a costly and lengthly experimental procedure and, on the contrary, Π_2 is the result of a quick and easy physico-chemical measure. A concrete case may be: $\Pi_1 \equiv$ biological activity and $\Pi_2 \equiv$ chromatographic index. Then, suppose that two functions $\Phi = \{\varphi_1, \varphi_2\}$, built in the sense of equations (1) can be obtained. So far an unknown molecule, i, of M_u

$$\Pi_{i1} \sim Z_{i1} = \varphi_1(P_{iu})$$
$$\Pi_{i2} \sim Z_{i2} = \varphi_2(P_{iu}) \qquad (5)$$

But nothing is against the existence of a more accurate function

$$\Pi_1 = \Theta \quad (\Pi_2) \qquad (6)$$

which provides a way to obtain costly experimental results in a very simple manner. So, let's suppose that function Θ exists. In that case, a good synthetic planning will be to start estimating Π_2 with φ_2 and then use Θ instead of φ_1 to approximate Π_1. Once a new structure is reached, Π_2 may be checked experimentally against the value Z_{i2} and function Θ used to obtain an estimate of Π_1.

In this case the property Π_2 acts as a "filter" in order to optimize the experimental part of a new synthetic process.

Many other interesting plannings may be thought of. Let's suppose now that Π_1 and Π_2 are both attached to some biological activities. Optimal drug designs can be based on the whole set Π. For example, obtain maximal Π_1 submitted to a minimal Π_2, or given an activity threshold τ, then $\Pi_2 < \tau$.

From this example we can see that the concept of correspondence between molecular structure and n-dimensional metric enlarges to an unknown extent the QSAR possibilities. But molecular structure imposes the law, to n-dimensional spaces. One of these laws is disconnection: when representing molecules in a geometrical way one cannot expect to find a connected surface or volume where each point can be associated to a given structure. The chemical structure itself makes each molecule behave, from a geometrical optic, as an isolated point in n-dimensional space. One can expect to find neighbours to a given structure, but it is unphysical to think of a possible filling of the gaps between points. The topological concept of connection is related to the idea of a set that looks like being piece less, and this cannot be the situation described in the molecular spaces. The principal property of these spaces is disconnection.

So, if one is lucky enough to find a beautiful set of functions Φ, one must be extremely careful about them. Despite of the possible form, they cannot be everywhere definite. For example, optimal points computed on such functions may not be necessarily attached to any real or reachable structure, that is, synthetizable and sufficiently stable to be of practical use.

PARAMETERS

As we discussed above, parameters constitute the building blocks of QSAR. Without parameters no theoretical predictive framework is possible. In the available literature on QSAR, the parameters to construct the function set Φ are present in increasing amounts. Three main sources can be defined, providing the rough classification scheme we present here:

a) Empirical parameters, whose origin is commonly a given experimental variable, are well known by organic chemists. The most conspicuous one may be the Hammet's σ, an additive artifact used in many places (March, 1977). Along with this parameter one must compulsively quote the Hansch's π as a tool to reproduce partition coefficients between two phases (Hansch, 1969). The main idea attached to this parameter is that activity in many circumstances will depend on the diverse molecular abilities to reach aqueous physiological phases traversing lipid barriers. The parameter π has been used in large amounts of data, for a recent review the monography of Martin (1978) is recommended. In the same place, other parameters of this kind are also described, they usually are used to add some environment effects which cannot be given by π alone.

b) Structural parameters, one can call in this manner all the

parameters obtained by direct observation of the set M or by mathematical manipulations based on the structure of its elements. A set of such molecular parameters can be constructed by simply inspecting the substitution sites and the molecular substituents present. This process may generate a set of variable vectors with binary valued elements, using the simple fact that a given molecular feature may be present or absent. This kind of parameter can be used in taxonomic or pattern recognition procedures.

Another set of parameters which may be classified in the same group are those related with graph theory, a mathematical tool associated to molecular structure through the bonds drawn by chemists when they try to picture molecules. The most known and successful parameter in this cathegory was developed by Kier (1976), named connectivity and symbolized by χ. As it seems to happen with all parameter families a wide variety of connectivity variants has been developed after the initial definition. Other less competitive ideas had also been defined, with very good results (Dubois, 1966).

c) Quantum Mechanical parameters, which consitute perhaps the widest pool of available theoretical information on molecules. Quantum Chemistry has developed a large panoply of variables which can be extracted from a not lesser reservoir of diverse methods. Beside the possible direct use of Quantum Chemistry to study biologically related molecular interactions, conformations and reaction hypersurfaces, indirect use of the wavefunction information may result in a quite convenient source in QSAR calculations. In fact, many authors had recourse to it (Cammarata, 1971; Del Re , 1963; Firpo, 1975; Otto, 1979; Streitwieser, 1961), and even if the potential user is not very fond of costly "ab initio" methods, a good deal of information may be obtained from simpler procedures.

A great amount of research, comparable to other analysis using parameters of a) and b) types, has been done with the aid of atomic charges coming from naive methods, easily computable with inexpensive programs (Carbó, 1970; Del Re, 1958; Pullman, 1963); other parameters related with MO theory are the superdelocalizabilities described by Fukui (Higasi, 1965), dipole moments, HOMO and LUMO energies, ... and a large list of other possible candidates.

May be the simplest picture of a relationship between structure and activity can be given by an approximate electrostatic interaction energy expression:

$$E_{RM} = \sum_{I \in R} \sum_{J \in M} \frac{Q_I^R \, Q_J^M}{r_{IJ}} \qquad (7)$$

between a receptor (R) and a given molecule (M). If for a given molecular set there appears to be a unique receptor . and activity is related with interaction energy, then (Carbó, 1977):

$$A_M \propto E_{RM} = \sum_{J \in M} a_J Q_J^M \, , \qquad (8)$$

the coefficients $\{a_J\}$, collecting the supposed constant interaction features. Volume and solvation effects may influence activity. They may be introduced in the equation (8), through other parameters as usually is being done in the previous parameter groups a) and b).

Quantum Mechanical parameters are related to a molecular wavefunction or to a first order density function, and the last one can be used directly in QSAR calculations. This use introduces a new feature into molecular spaces: if molecules can be described by a function, then we can also have infinite dimensional spaces at our disposal.

MOLECULAR ENGINEERING

At the moment we can make a rough draft of what we want to call Molecular Engineering: the n-dimensional molecular spaces allow us to lodge properties and parameters; with some luck and a good deal of chemical intuition we can have a molecular family with the pertinent properties and - what is even more difficult - the right parameters. Every molecule can be imagined as a point in the n-dimensional space whose directions are parameters and properties. So, moving in the right path, we can find the convenient point, the desired molecule. But the right path, if it exists, is supported by the correct relationship between properties and parameters. One has only to find this relationship.

This may be a difficult task, but, fortunately we have a large variety of tools - some of them leading to similar results - to do it.

METHODOLOGICAL TOOLS

The wide variety of techniques for dealing at the same time with n variables have the common name of Multivariate Analysis. Those techniques are the appropriate tools to work in the molecular spaces.

Multivariate Analysis, higly developed in the past, has become at present a usual computational device, with the aid of well known systems; as an example see Dixon (1977) and Nie (1975). From the available techniques the most used is multivariate correlation, which fills as leading procedure the whole set of QSAR work, and has inspired interesting analysis on the subject, (Martin, 1979; Otto, 1979; Topliss, 1979; Wooldridge, 1980). Multivariate correlation possess a large group of companion techniques - see, for example the review of Martin (1979) with specific applications to QSAR - interrelated through a well known device: the correlation matrix, whose elements connect all the variables used in single population multivariate analysis. Correlation matrices are simply computed once the molecular representation matrix $\underline{X}$ is known, and constructed in such a way as that the elements $\{x_{ij}\}$ represent the value of the jth molecular descriptor - property or parameter - for the ith molecule. In practice one can make a partition of $\underline{X}$ among its columns $\{x_j\}$, then one can write: $\underline{X} = (x_1, x_2, \ldots x_n)$.

For each column one can suppose that an origin shift and a scaling has been previously performed, such that each column bears zero mean and unit variance. If this is fulfilled, the correlation matrix may be computed as

$$\underline{R} = \underline{X}^T \underline{X} = \sum_j x_j^T x_j \ , \qquad (9)$$

as always we will obtain $r_{ii} = 1$, $\forall i$. An orthogonal set of molecular parameters will give $\underline{R} = \underline{I}$. Linear correlation functions between molecular properties and parameters is a straightforward matter once the matrix R is known, the procedure is too well described in many places (see, for example: Labart (1975), where a general review on Multivariate Analysis is given) to be repeated here. The same happens with the principal components technique, based on the eigenspace attached to the matrix $\underline{R}$. One of the main advantages of principal components is the possible use of the first $\underline{R}$ eigenvectors to represent the molecular points projected into two dimensional spaces. The technique, thus, allows a human visualization of the n-dimensional description with the minimal distortion. Figure 1, gives as a short example, the map obtained using principal components analysis of a phenol family, molecular parameters have been taken from empirical quantum mechanical calculations. The family has already been studied by Hall (1978) and by Mercier (1979) using structural parameters, but only from a correlation point of view

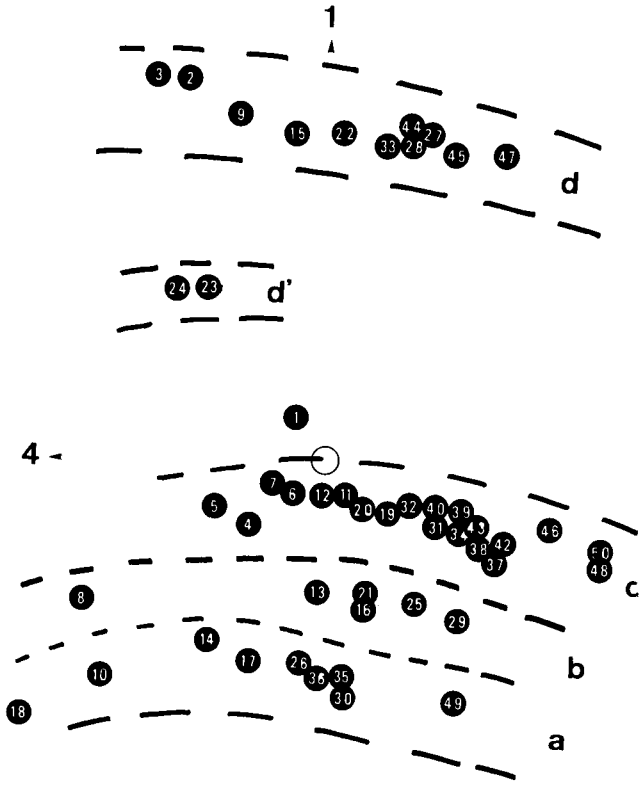

Figure 1. Principal components plot of a phenol family

Figure 1 shows the presence of various subsets corresponding each one to a main substituent characteristic. From the simple picture the features of the phenol set activity can be easily visualized. Numbering corresponds to the list given by Mercier (1979) and (1) corresponds to phenol molecule. Four main groups can be seen in the figure, each one corresponding to a given phenol substitution. Groups a), b) and c) describe para halogen substitution, and ortho hydrocarbon chain, they also differ on methyl substitutions: a) 3-5 dimethyl, b) 5-methyl, c) none. Group d) has ortho halogen substitution, para hydrocarbon chain and no methyl substitution. Elements (23) and (24) are separated from the group due to the presence of methyl substitution as in a). The most active compounds are those located in the right hand side of the figure for each group. This corresponds to a larger hydrocarbon chain in any group. It is worth noticing that compounds (46), (48) and (50) are found together in a precise region of the figure and correspond to the most active molecules.

The information provided by principal components analysis has been used to construct a linear function of the form

$$\log PC = -0.928 + 0.0521E + 2.309Q_2 + 4.082Q_4 \qquad (10)$$

with $F(3,44) = 208$; $n = 48$; $r = 0.966$;

where Q_2, Q_4 are charges on ortho and para positions, and E an energy term, which acts as a structural or molecular volume parameter. These quantities have been computed with Del Re (1958) method, modified by one of us (Carbó, 1970). This correlation can be amerliorated by introducing square terms, which also can be used for drug design optimization purposes. Introduction of E^2, Q_2^2, Q_4^2 and the cross term $Q_2 \cdot Q_4$ ameliorates the overall description ($r = 0.978$) and gives a definite negative hessian matrix, which means that it may be possible that there exists a maximum activity optimal structure. Although one must be quite cautious about extrapolations of this kind, the obtained results when varying the hydrocarbon chain, provides results comparable to the previously reported by Mercier (1979), using a quite different topological approach, though the side chain size is predicted here to be somewhat larger.

But there are other techniques at our disposal in order to deal with QSAR problems. Recent work: Chen (1979), Henry (1979), Jurs (1979), Moriguchi (1980) provides evidence on the excellent perspectives which can be attached to computer bound drug design.

PATTERN RECOGNITION

Pattern recognition has evolved into a very appealing mathematical structure - see, for example: Fu (1976), Tou (1977) - which has already been used in QSAR by Cammarata (1976), Menon (1977), Kirschner (1979) and Yuan(1980), and which must be related with Taxonomical techniques as described in the review of Sneath(1973).

As a final example of the possibilities associated with techniques other than multivariate correlation, we will briefly describe some recent development (Carbó, 1980) which is based on molecular density

functions and pretends to answer to the question: how similar is a molecule to another ? Given two molecular structures and knowing their respective density functions ρ_A and ρ_B, a similarity measure between both functions can be defined as

$$r_{AB} = \int \rho_A \rho_B \, dr \, / N_A N_B \qquad (11)$$

where $N_A^2 = \int {}^2_A \, dr$ and a similar expression holds for N_B. The value of $r_{AB} \in [0,1]$, and as it approaches 1 more one can say that $A \equiv B$. Given a molecular family one can compute a matrix $\underline{R} = \{r_{IJ}\}$ whose elements may be considered as correlation like coefficients. Comparing this matrix with the previously defined in Eq. (9) one must notice that instead of variables one is facing elements of the molecular set as dimension. On this matrix one can use minimal spanning tree algorithms or use other $\underline{R}$ matrix properties in order to get information on molecular space.

Table 2 presents the correlation matrix obtained with the set of five hexane isomers. From Table 2 one can draw a minimum spanning tree, remembering

Table 2. R matrix for hexane isomers

	A	B	C	D
B	.8745			
C	.8738	.8733		
D	.7396	.8744	.8731	
E	.7296	.7300	.8708	.8736

Key

A 2,2-dimethyl butane
B 2,3-dimethyl-butane
C 2-methylpentane
D 3-methylpentane
E Hexane

that the closest situation corresponds to the biggest value of r_{IJ}. The procedure gives the sequence A(C) - B - D - E, which fits very well the trend of the whole molecular properties tested, on the contrary of the tree obtained by Randic (1979), having the sequence A - C(E) - D - B, and which do not fit all observed sequences Randic (1978).

THE FUTURE OF QSAR

It is not difficult to predict sophisticated extensions and quick development of Molecular Engineering techniques. A recent work (Gund et. al., 1980) can shed light on some of the future possibilities, it is out of doubt that computer graphics and interactive computational systems will aid to transform QSAR into a more general framework. Another possible development should be related with the often missing link between QSAR theoretical results and practice. From one part the Learning Process structure developed in recent years (Simon, 1976) from the other the impact of Computers in Organic Synthesis (Bersohn and Esack, 1976) in particular and in Chemistry (Ugi et.al., 1979) in general, whoseevolution has been parallel to QSAR show the interest to collect all the available ideas in order to obtain a powerful means to attack purposeful synthesis of new molecules. It is certainly sure that other techniques and points of view, not mentioned here, already exist and are waiting to be included into the path of QSAR developing

towards Molecular Engineering.

Let us hope that at the beginning of the next decade our knowledge of molecular optimal design will really have transformed QSAR into an almost exact Science.

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Towards More Realistic Computer Simulations in Theoretical Molecular Biology

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Quantum-chemical computations on biological systems have been, and often are, characterized by the acceptance of rather gross approximations, due to the diffuse opinion that for complex chemical systems accurate computations are unfeasible. During the last decade, however, sufficient evidence has been accumulated to reject the above opinion. Below are listed a few significant areas where progress has been achieved.

Electrostatic Potential Maps Versus Iso-energy Maps

It is customary to discuss the reactivity of two interacting molecules, A and B, by computing the electrostatic potential of A due to B, where B is approximated by a set of point charges, and A is approximated by some basis set. The advantage of the method is related to its computational simplicity (Scrocco and Tomasi, 1973). The drawbacks of the method are: 1) basis set dependency, 2) breakdown of the electrostatic approximation at short interaction distances, 3) possibility of breakdown of the electrostatic approximation even at equilibrium distances, 4) partial or total neglect of exchange, charge transfer, polarization and induction effects, and 5) practical impossibility to use the method for the case of either A or B larger than 30 to 60 atoms. In addition, in practice, molecule B is often simulated by a simple point charge, thus describing the interaction in a very unspecific manner. It has been proposed to use iso-energy maps obtained from two-body analytical potentials, since none of the above five limitations affect the iso-energy maps. Chemical systems composed of several hundred atoms have been analyzed with iso-energy maps; a library of atom-atom pair potential is available (Corongiu and Clementi (1978); Clementi (1976); Clementi (1980)).

Characterization of an Atom in a Molecule

The traditional classification of an atom in a molecule is related to its valence (and hybridization). Thus, for example, years ago about three different types of carbon atoms had been assumed to be sufficient for the classification of all carbon atoms in the naturally oc-

curing amino-acids (Momany, Curruthers, McGuire and Sheraga, 1974). We have proposed to classify atoms not only in terms of hybridization but also in terms of their distribution of net charges (Mulliken, 1955) and molecular orbital valence state energy (Clementi, 1976, 1980). In this way, for example, about 13 different carbon atoms have been found necessary to describe the carbon atoms in amino-acids. This refined classification is essential to ensure transferability of two-body potentials, and therefore, to ensure a reliable basis for the use of iso-energy maps.

Basis Set Superposition Error

The use of a very small basis set (like STO-3G) can bring about a very large error in the computation of intermolecular interactions. (Errors ranging from 30% up to 150% seem to characterize quantum-chemical computations in biochemical systems.) As we know, the counter-poise method (Boys and Bernardi, 1970) can nearly eliminate the basis set superposition error. (This method seems to be nearly unknown to many quantum-biochemists.) It is noted that the use of an extended basis set, while substancially less exposed to basis set superposition error, has the known drawback of requiring much computer time. On the other hand, the counter-posed method is computationally fast (Kolos, 1979; Kolos, Corongiu, Clementi, 1980).

Direct Computations of the Correlation Energy Correction

The Hartree-Fock method yields wave functions that are un-correlated; binding energies from un-correlated wave functions can be grossly in error (up to 50% to over 100%). The correlation energy correction can in general be computed by using many determinants (configuration interaction), a technique, however, that becomes rapidly unyielding for large chemical systems. On the other hand, it has been known for the past forty years that the correlation energy correction can be approximated as a function of the Hartree-Fock density (Wigner, 1934). A very simple functional has been proposed and tested for diatomic molecules (Clementi, 1965, 1972; Lie and Clementi, 1979), as well as for predicting the ionization potentials and the electron affinity (Clementi, 1980). Therefore, the traditional neglect to even estimate the correlation energy correction seems to be less and less justified.

Indirect Computations of the Inter-molecular Correlation Energy Correction

A significant fraction of the intermolecular interaction energy can be associated with the dispersion energy, which is not described by the Hartree-Fock method. It has recently been shown (Kolos, 1979; Kolos, Ranghino, Novaro and Clementi, 1980; Clementi, Kolos, Lie, Ranghino, 1980) that by using minimal basis sets, the counter-poise method and a simple semi-empirical expression for the dispersion energy based on the perturbation theory, one can obtain intermolecular interaction energies about as accurate as those obtained by using extended basis set and thousands of determinants. It is stressed that the computational effort needed in the above perturbation technique decreases several hundred times. Again, we con-

clude (as above) that the traditional neglect in biochemical computations of the electronic correlation energy correction has less and less justification.

Induction Energy Correction

The direct computation of a system of n interacting small molecules, is computationally expensive, because of the many degrees of freedom in the system. Indirect computations, by two-body potentials, are relatively inexpensive, yet are relatively limited in their predictive value because of the non-additivity of the two-body potentials. A notable fraction of the non-additivity correction is represented by the induction energy. The use of minimal basis sets, the counterpoise method and a simple relation representing the induction correction allows one to obtain in a simple way three- and many-body corrections (Clementi, Kistenmacher, Kolos, and Romano, 1980).

Static Reaction Field

Due to the complexity of the computer simulations it is customary in biological computations of interacting large chemical systems (like in enzymatic reactions, mutagenetic effects, protein synthesis, etc.) to neglect most of the atoms that constitute the chemical system and to consider only a very few atoms. It has been shown (Bolis and colleagues, 1978) that entalpy variations of a complex system (like papaine interacting with a substrate) can be realistically simulated by retaining all the atoms of the system and by decomposing the problem into macro-and micro-steps. In the macro-steps, empirical potentials are used to determine the overall geometry of the macrosystem. In the micro-steps, ab-initio quantum-chemical computations are performed for the atoms at the reaction site. The reaction field inclusion in the micro-steps is essential. For example, in their papaine study, by neglecting the reaction field - the traditional way to follow biochemical reactions in quantum-chemical computations - at a given reaction step, a barrier of about 18 Kcal/mol was computed; inclusion of the reaction field, however, did lower this barrier to about zero Kcal/mol. It can be safely stated that neglect of the reaction field deprives many quantum-chemical computations on a biological system of a basic reason for expecting a quantitative and at times even qualitative value in the predictions. In the above quoted study (Bolis and colleagues, 1978), only the static component of the reaction field has been considered. The time-dependent fluctuations of the reaction field are even less understood, but could represent an essential factor in biological reactions (Clementi, 1980).

Temperature Induced Degeneracy

Let us consider a system composed of n small molecules interacting with a large molecule; an example is a biological solute in water at room temperature. As known, there are many configurations for such systems, all very near in energy (namely all those configurations that differ by an energy amount of the order of KT). By a variation in temperature, the number of nearly degenerate conformations will

change (see for example Clementi and Corongiu, 1980). This notion is well established in solution studies; however, when we consider much of the recent literature on solvent effects in biological systems we realize that temperature statistics are very often ignored. As known, reliable methods like Monte Carlo (Metropolis and colleagues, 1953) and molecular dynamics (Alder and Wainwright, 1959) have long since been introduced to deal with such problems. Monte Carlo applications to solutions containing amino-acids (Romano and Clementi, 1980), simple molecules like CH_4 (Scheraga, 1978), or complex molecules like enzymes (Ranghino and Clementi, 1978; Clementi, Corongiu, Jonsson and Romano, 1979), and nucleic acids (Clementi and Corongiu, 1979) have been reported from relatively few groups.

Entropy and Free Energy Computations

As known the entropy variations can be a basic parameter to predict reactivity. However, in much of today's biomolecular computer simulations, entropy is seldom investigated or even mentioned. It must be stressed that since most biomolecular reactions occur in solution, entropy might often be a dominant term. It is expected that accurate simulations on entropy and free energy will lag for some time; indeed theoretical determinations of the entropy contribution to the free energy by computer simulations are still very expensive (Mezei, Swaminathan, and Beveridge, 1978; Slanina, 1979; Romano and Singer, 1979). Solvation entropy is only one of the many aspects; vibrational activation entropy has been suggested as a very important contribution to enzyme catalysis (Cook and McKenna, 1974).

Time Dependent Simulations

The temporal evolution is one of the most important characterizations of a biological system; indeed outside the temporal frame, problems like stability, instability, or energy dissipation, etc., lose much of their meaning. At least three main avenues are available. At the quantum-mechanical level, scattering theory could be used; however, the complexity of biological systems is such as to make it unlikely that scattering theory based formalisms will be of much use. At the chemical level, molecular dynamics can be fruitfully used, provided reasonable pair-potentials are selected. Molecular dynamics computations are, however, limited by a) the very short interval of time that can be simulated and, b) by the limited number of atoms or molecules that can be described. Fluid dynamical simulations likely constitute the area where one could safely expect the most rewarding progresses. One of the reasons for the above statement is that theoretical molecular biology needs to be linked to theoretical cellular biology (LeFever and Goldbeter, eds., 1976; Nicolis and Prigogine, 1977) and, slowly, there is some progress in this direction.

In conclusion, one can note that the field of computations for biological systems is continuously expanding in the number and types of applications. This positive aspect, however, needs to be balanced by the realization that too often theoretical computations are carried out at a rather unrealistic level and most questionable approximations are often uncritically retained. Biochemical computations

are often accepted for publication despite the use of methods which lag by about 20 years, relative to those of statistical mechanics, and by about 10 to 15 years, relative to decent quantum-chemical computations. The often adopted justification is the cost in computer time; however, fast, correct and available methods are often ignored. The "mentality" that each field (including quantum-biology) must develop its own techniques seems at present to have relatively few active supporters. A detailed analyses and numerous biochemical examples concerning this "new" mentality is available (Clementi, 1980). In that quoted work we have shown that by a combined use of quantum mechanics and statistical mechanics, the "matrix" of biochemical problems reported in the figure can be simulated with realistic rather than very gross approximations. In the last six years, the main emphasis has been on few applications concerning the first column of the matrix, that deals with solutions and solvents. The recent availability of pair potentials to describe interactions of any amino-acid with an amino-acid, as well as ions with amino-acids and nucleic-acids (Clementi and Corongiu, 1980), opens the way to realistic applications in the remaining columns of the "matrix", and establishes a strong base to increase the matrix size.

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AB Initio Quantum Chemical Approach to the Antibiotic Activity of ß-Lactams

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ABSTRACT

Quantum chemical investigations of the base-catalysed breaking of the endocyclic amide bond of some β-lactams are presented and discussed through ab initio Hartree-Fock calculations of the potential energy surfaces of the β-lactam + OH^-, CH_3O-β-lactam + OH^-, and 3-cephem + OH^- reactions. The initial attack of the nucleophile is similar for each reaction and gives a tetrahedral intermediate. The breaking of the amide bond occurs with a reaction barrier which is remarkably lower in 3-cephem than in the monocyclic β-lactams owing to the electron-withdrawing effect of the enamine group. The product is stabilized by an intramolecular hydrogen bond and its energy depends on intramolecular steric interactions. The theoretical results are correlated with some experimental data on the antibiotic activity of β-lactams.

KEYWORDS

Quantum chemical calculations; ab initio Hartree-Fock; β-lactams; amide bond breaking; antibiotic activity.

INTRODUCTION

The last stages of the peptidoglycan synthesis of the bacterial cell walls are catalysed by DD - carboxypeptidase and transpeptidase enzymes through the nucleophilic attack of the enzyme to the amide carbon of the terminal D-Ala - D-Ala group of a peptide chain and the following breaking of the amide bond; the resulting peptide-enzyme complex is unstable and easily reacts with another peptide chain to give peptidoglycan and the free enzyme. The β-lactam antibiotics react in a similar way with the enzymes through their endocyclic amide bond, but the corresponding antibiotic-enzyme complex is very stable so that the peptidoglycan synthesis is inhibited (Flynn, 1972; Blumberg and Strominger, 1974; Ghuysen and others, 1980).

A theoretical study of a reaction can be carried out through quantum-chemical methods in the Born-Oppenheimer approximation (1927) by calculating the electronic energy at different nuclear geometries and so obtaining the potential energy surface and reaction paths of the nuclei in the average electronic field. Since the base-

catalysed breaking of the C-N bond of the peptide or of the β-lactam ring is the central stage of both the reactions of these substrates with the enzymes, we present in this paper the current state of ab initio quantum-chemical studies of this bond breaking in β-lactams.

PROCEDURE

Physical Models

The potential energy surfaces for the β-lactam + OH^-, CH_3O-β-lactam + OH^- and 3-cephem + OH^- reactions have been recently investigated (Petrongolo and others, 1980a, 1980b, 1980c). General information on the amide-bond breaking in β-lactams can be obtained from the study of the first reaction, while the effcts of the 7α-methoxy substitution and of the dihydrothiazine ring of Δ^3 active cephalosporins have been investigated in the second and third reaction, respectively. Finally the OH^- ion has been chosen as nucleophile since it is the attacking species in the base-catalysed hydrolysis of β-lactams and it is also a model of the serine OH group which is probably an active site of serine DD-carboxypeptidases (Ghuysen and others, 1980).

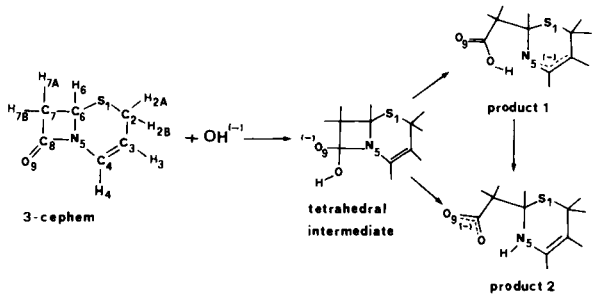

Fig. 1. Tetrahedral mechanism of the 3-cephem + OH^- reaction.

Fig. 1 shows the investigated mechanism which is characterized first by the formation of a tetrahedral intermediate and then by the C_8-N_5 breaking which can yield two products, with or without H transfer from OH^- to N_5; this tetrahedral mechanism is very common for amides and it is preferred to the S_N2 one (March, 1968). In conclusion, the physical models have been chosen on the basis of the experimental evidence and of the complexity of the problem to be studied through ab initio methods; the comparison between these reactions is essential to discriminate between the effect of the β-lactam ring and that of the dihydrothiazine ring, and between the role of the 3-cephem core and that of its substituents.

Details of the Calculations

Ab initio Hartree-Fock calculations (Roothaan, 1951) have been carried out with the STO-3G basis set (Hehre and others, 1969) for β-lactam + OH^- and with a better 9s6p/7s3p/3s basis set (Petrongolo and others, 1980b) for all three reactions. A complete determination of these multidimensional surfaces (48 dimensions for 3-cephem + OH^-) is impossible at the present state of art of ab initio calculations and it is also unnecessary for the study of the mechanism of Fig.1. We have thus considered three portions of the overall surfaces which correspond to three bond structures of the H atom of OH^-: structure a with H bound to O and the dihedral angle $<HOC_8O_9$ or $<HOC_8N_5 = 0°$ (the intermediate and the product 1 have this structure); structure b with H partially bound to O and N_5 and $<HOC_8N_5 = 0°$ (corresponding to a partial H transfer from O to N_5); structure c with H bound to N_5 and $<HN_5C_8O$ or $<HN_5C_8O_9 = 0°$ (the product 2 has this structure). This method gives good results with a moderate computational effort as a test on the formamide + OH^- reaction has shown (Petrongolo and others, 1980a).

The geometries of the three substrates have been partially derived from the X-ray structure of cephaloridine (Sweet and Dahl, 1970) and by placing CH_3O on the 7α position of the ring; the β-lactam ring is therefore planar or nearly planar and the N_5 atom is pyramidal. Eleven geometrical parameters have been varied for each bond structure: the bond angle $\alpha = <C_7C_6N_5 = <C_8C_7C_6$, the dihedral angle $\tau = <C_8C_7C_6N_5$ and the nine coordinates of the O_9, O and H atoms; the angles α and τ are the guide parameters of the reaction since both describe the C_8-N_5 breaking. On the whole ∿170 and ∿50 points of the surface of β-lactam + OH^- have been calculated with the STO-3G and 7s3p/3s basis, respectively; ∿50 points have been calculated for 3-cephem + OH^- with the 9s6p/7s3p/3s basis, and only the most significant points of the reaction path of CH_3O-β-lactam + OH^- have been calculated with the 7s3p/3s basis.

REACTION SURFACES

The potential energy surfaces of the β-lactam + OH^- and 3-cephem + OH^- reactions are shown in Figs. 2-4. A detailed analysis of these surfaces has been reported elsewhere (Petrongolo and others, 1980a, 1980b) so that we here discuss only the most important results which are summarized in Figs. 5-8 and in Tables 1 and 2 and which report some reaction paths.

From the Reagents to the Intermediate

The OH^- attack to both α and β faces of C_8 has been considered. In both cases the nucleophile approaches without energy barrier and the potential energy decreases till the formation of a tetrahedral intermediate which is strongly stabilized with respect to the reagents owing to the formation of a C_8-O covalent bond. The structure of the intermediate for the α-face attack to 3-cephem is shown in Fig. 7 and it is characterized by tetrahedral C_8 and N_5 atoms, by a O-H--O_9 hydrogen bond (structure a with $<HOC_8O_9 = 0°$), and by τ equal to that of the substrate. The OH^- attack to the β-face of the ring yields a structure which is ∿8 kcal mol^{-1} less stable than the intermediate, while a test carried out by clamping N_5 to a sp^2 hybridization in the β-lactam + OH^- reaction gives another structure with energy ∿16 kcal mol^{-1} greater than that of the intermediate. Both these results are due to repulsive interactions between the OH group and the H_5 atom of the monocyclic β-lactam or the enamine group of 3-cephem. On the other hand, the attack of OH^- to β-lactam with a planar

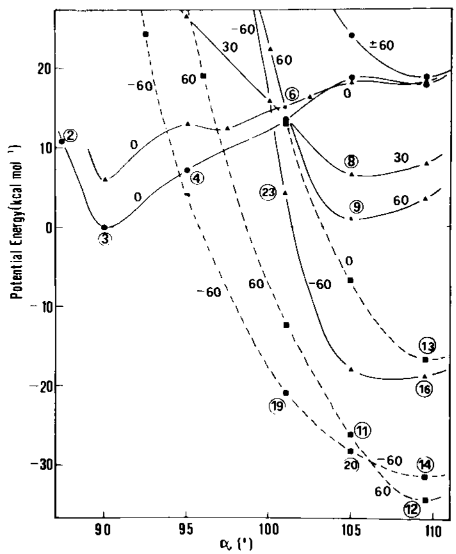

Fig. 2. STO-3G potential energy surface of the β-lactam + OH^- reaction. Solid curves: structure a with $<HOC_8O_9$ = 0° (circles) and $<HOC_8N_5$ = 0° (triangles). Dashed curves: structure b (stars) and structure c (squares). The τ values and the points of the reaction paths are also shown.

N_5 atom (which is ∿5 kcal mol^{-1} more stable than β-lactam with pyramidal N_5) yields immediately a N_5 pyramidalization in the initial stage of the interaction, much before the formation of the intermediate; therefore the N_5 hybridization of monocyclic β-lactams can easily change from sp^2 to sp^3 during the nucleophilic attack. Finally, the S_N2 mechanism can be excluded just on the basis of the formation of an intermediate which corresponds to a minimum of the reaction path.

In this first portion of the reaction path no significant differences have been found between the investigated reactions, including also formamide + OH^- (Petrongolo and others, 1980a, 1980b): the 9s6p/7s3p/3s stabilization energy of the intermediate with respect the reagents is ∿108, 112 and 116 kcal mol^{-1} for β-lactam + OH^-, CH_3O-β-lactam + OH^- and 3-cephem + OH^-, respectively, and this value rises to ∿121 kcal mol^{-1} for the first reaction by using the STO-3G basis set.

From the Intermediate to the Barrier

This reaction stage is mainly characterized by a partial breaking of the amide bond of the β-lactam ring till C_8-N_5 distances ≈2.1-2.2 Å. This bond breaking occurs without torsion of the ring, with H always bound to O, and with a torsion of the O-H bond about the O-C_8 one from $<HOC_8O_9$ = 0° to $<HOC_8N_5$ = 0°. Owing to these geometrical changes the potential energy increases from the value of the intermediate to a maximum corresponding to the reaction barrier (point 6 of the reaction path; see Fig. 7 for the structure of this point for the 3-cephem + OH^- reaction).

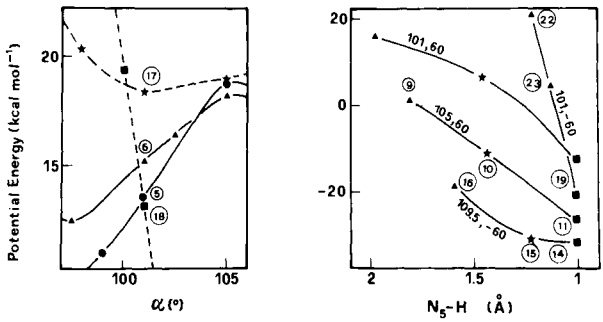

Fig. 3. Particular views of the STO-3G potential energy surface of the β-lactam + OH^- reaction. On the left: barrier region; on the right: H transfer.

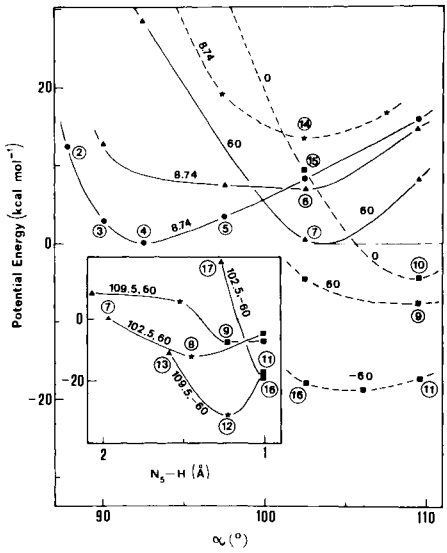

Fig. 4. 9s6p/7s3p/3s potential energy surface of the 3-cephem + OH^- reaction. The H transfer is shown in the small insert.

The barrier heights of the β-lactam + OH^- and CH_3O-β-lactam + OH^- reactions are very similar: ∿15 kcal mol^{-1} for the first reaction, by using both basis sets, and ∿16 kcal mol^{-1} for the second reaction. On the contrary the breaking of the amide bond of the intermediate of 3-cephem + OH^- occurs with a barrier height about equal to 7 kcal mol^{-1}, this value being remarkably lower than that found for the other investigated substrates. This result points out the labile character of the amide bond

of 3-cephem with respect to monocyclic β-lactams and it is due to the effect of the dihydrothiazine ring, as the analysis of the following section will show.

TABLE 1 STO-3G Reaction Paths of β-Lactam + OH^- a)

Path	Point	Description	Struct.	α	τ	C_8-N_5	N_5-H	Energy
I	1	reagents	a	86.16 d)	0	1.37	∞	120.8
	2		a	87.5	0	1.44	3.03	10.7
	3	tetrahedral intermediate	a	90	0	1.57	3.15	0
	4		a	95	0	1.83	3.37	7.2
	5		a	101	0	2.13	3.37	13.5
	6	reaction barrier	a	101	0	2.13	2.40	15.2
	7		a	101	30	2.26	2.01	13.9
	8		a	105	30	2.45	1.92	6.8
	9		a	105	60	2.73	1.94	1.1
	10		b	105	60	2.73	1.44	-11.1
	11		c	105	60	2.73	1.01	-26.2
	12	product 2, τ = 60°	c	109.5	60	2.91	1.01	-34.1
	13	torsional barrier	c	109.5	0	2.55	1.01	-16.6
	14	product 2, τ = -60°	c	109.5	-60	2.91	1.01	-31.4
	15		b	109.5	-60	2.91	1.23	-31.0
	16		a	109.5	-60	2.91	1.59	-18.8
II b)	17	barrier; path II	b	101	0	2.13	1.32	18.4
	18		c	101	0	2.13	1.01	13.1
	19		c	101	-60	2.58	1.01	-20.9
	20		c	105	-60	2.73	1.01	-28.1
III c)	21		a	101	0	2.13	2.38	16.3
	22	barrier; path III	a	101	-60	2.58	1.22	21.1
	23		a	101	-60	2.58	1.14	4.4

a) Lengths in Å, angles in degrees and energies in kcal mol^{-1} with respect to point 3 whose total energy is equal to -316.87378 hartree (1 hartree = 627.506 kcal mol^{-1}).
b) Path II starts with points 1-6 and continues with points 17-20 and 14.
c) Path III starts with the points 1-6 and continues with points 21-23, 19, 20 and 14.
d) Mean value between $<C_7C_6N_5$ = 86.42° and $<C_8C_7C_6$ = 85.89°.

From the Barrier to the Product

After the point 6 several reaction paths are possible, all corresponding to the complete breaking of the C_8-N_5 bond, to a partial H transfer from O to N_5, and to complex conformational changes of the system.

(1) The minimum-energy path of each reaction begins with a torsion of the β-lactam ring about the C_7-C_6 bond and on the same side of the dihydrothiazine ring, till τ ≃ 60°, whereas the α bond angle and the O-H bond length are equal to the values of the point 6. This torsion yields a further breaking of the C_8-N_5 bond and a decrease of the potential energy.

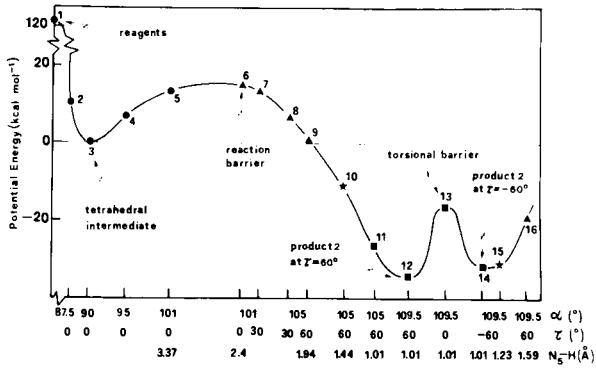

Fig. 5. STO-3G minimum-energy reaction path of β-lactam + OH^-.

TABLE 2 9s6p/7s3p/3s Reaction Paths of 3-Cephem + OH^- a)

Path	Point	Description	Struct.	α	τ	C_8-N_5	N_5-H	Energy
I	1	reagents	a	86.16 d)	8.74	1.39	∞	115.8
	2		a	87.82 e)	9.28	1.48	3.12	12.4
	3		a	90	8.74	1.59	3.09	3.0
	4	tetrahedral intermediate	a	92.5	8.74	1.71	3.18	0
	5		a	97.5	8.74	1.97	3.34	3.4
	6	reaction barrier	a	102.5	8.74	2.22	2.21	7.0
	7		a	102.5	60	2.64	1.97	0.5
	8	secondary minimum	b	102.5	60	2.64	1.46	-12.0
	9		c	109.5	60	2.92	1.23	-7.5
	10	torsional barrier	c	109.5	0	2.55	1.01	-4.5
	11		c	109.5	-60	2.91	1.01	-17.3
	12	product	b	109.5	-60	2.91	1.23	-30.9
	13		a	109.5	-60	2.91	1.59	-11.1
II b)	14	barrier; path II	b	102.5	8.74	2.22	1.63	13.6
	15		c	102.5	10.75	2.23	1.12	9.4
	16		c	102.5	-60	2.64	1.01	-18.3
III c)	17	barrier; path III	a	102.5	-60	2.64	1.27	18.7

a) Lengths in Å, angles in degrees and energies in kcal mol^{-1} with respect to point 4 whose total energy is equal to -830.70738 hartree (1 hartree = 627.506 kcal mol^{-1}).
b) Path II starts with points 1-6 and continues with points 14-16 and 12.
c) Path III starts with points 1-6 and continues with points 17, 16 and 12.
d) Mean value between $<C_7C_6N_5$ = 86.42° and $<C_6C_7C_8$ = 85.89°.
e) Mean value between $<C_7C_6N_5$ = 86.42° and $<C_8C_7C_6$ = 89.22°.

The β-lactam + OH^- system is more stabilized by the following α increase till ∿105°, by the H transfer from O to N_5, and finally by the complete C_8–N_5 breaking thus arriving at a minimum of the reaction path which corresponds to a first conformation of the product 2 of Fig. 1 with τ = 60° (point 12 with structure c). This point is stabilized by a N_5–H--O^- hydrogen bond (<HN_5C_8O = 0°) and is ∿34–36 kcal mol^{-1} below the intermediate, by using the two basis sets. The torsions of the COO^- group and of the ring of the point 12 occur in a concerted way with a torsional barrier at τ = 0°, and then the energy decreases till a second conformation of the product 2 with τ = -60° and <$HN_5C_8O_9$ = 0°. In this last reaction stage the differences between the two basis sets are noticeable (see Fig. 8). The STO-3G basis yields the point 14, ∿3 kcal mol^{-1} above the point 12, with H bound to N_5 (structure c) and an unsymmetric N_5–H--O_9^- hydrogen bond; instead the 7s3p/3s basis gives the point 15, ∿11 kcal mol^{-1} below the point 12, with H partially bound both to O and to N_5 (structure b) and a symmetric $[N_5$--H--$O]^-$ hydrogen bond. Since the 7s3p/3s results are confirmed by previous accurate studies (Clementi, 1967; Kollman and Allen, 1970, 1971), only this basis set have been used for the CH_3O-β-lactam + OH^- and 3-cephem + OH^- reactions. Finally we note that the product of CH_3O-β-lactam + OH^- reaction is ∿44 kcal mol^{-1} below the corresponding intermediate, in complete agreement with the β-lactam + OH^- results.

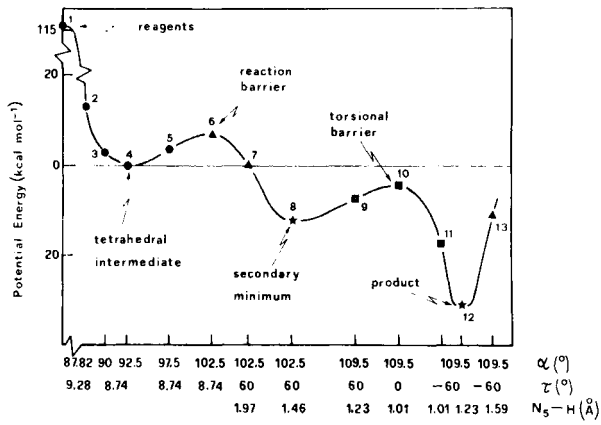

Fig. 6. 9s6p/7s3p/3s minimum-energy reaction path of 3-cephem + OH^-.

The final stage of the reaction path of 3-cephem + OH^- is somewhat different from that of the monocyclic β-lactams. The comparison between Tables 1 and 2 and between Figs. 5 and 6 shows that, after the initial torsion of the point 6 till τ = 60°, 3-cephem + OH^- is stabilized only by a partial H transfer from O to N_5 without increase of α and that a secondary minimum of the path is obtained (point 8, ∿12 kcal mol^{-1} below the intermediate) with the $[N_5$--H--$O]^-$ hydrogen bond previously discussed. Both the energy and the geometry of this secondary minimum are thus different from those of the point 12 of the β-lactam + OH^- reaction, although both these points

have $\tau = 60°$. After the secondary minimum the paths of the two reactions become more similar and the product of 3-cephem + OH^- (point 12, ∿31 kcal mol^{-1} below the intermediate) is again stabilized by the $[N_5\text{--}H\text{--}O]^-$ hydrogen bond, as Fig. 7 shows. We finally note that a steric hindrance of the enamine group of 3-cephem is pointed out by the destabilization of this reaction stage of 3-cephem + OH^- with respect to that of β-lactam + OH^-.

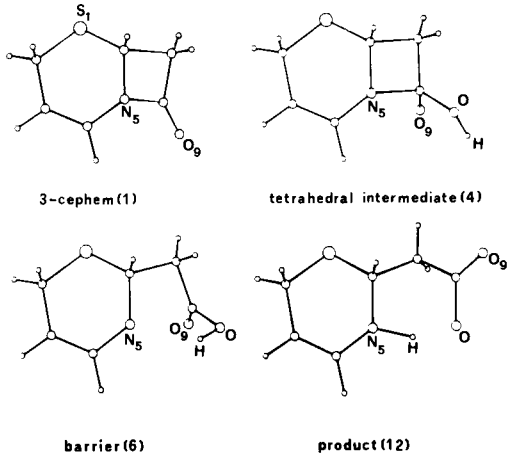

Fig. 7. Structures of 3-cephem, intermediate, barrier, and product of the 3-cephem + OH^- reaction.

(2) In a second reaction path (Tables 1 and 2) a partial H transfer from O to N_5 occurs with an energy increase with respect to the point 6, till the maximum of the path which is ∿18 and 14 kcal mol^{-1} above the intermediate of β-lactam + OH^- and 3-cephem + OH^-, respectively. The energy then decreases till the product owing to H movements concerted with the torsion and opening of the ring. A third reaction path is also possible with higher barrier (see Tables 1 and 2).

The previous results point out the similarities and differences between the investigated reactions and that the structure of the final product is intermediate between those shown in Fig. 1.

CNDO Calculations

The main points of the 3-cephem + OH^- reaction path have been also calculated by using the semiempirical CNDO/2 method (Pople and Beveridge, 1970). In this case however the results are completely different from the ab initio ones: with respect to the intermediate, the energies of the reagents, barrier and product are ∿222, 93 and 85 kcal mol^{-1}. We therefore think that ab initio calculations of small model systems are more reliable than CNDO/2 calculations of the complete molecules.

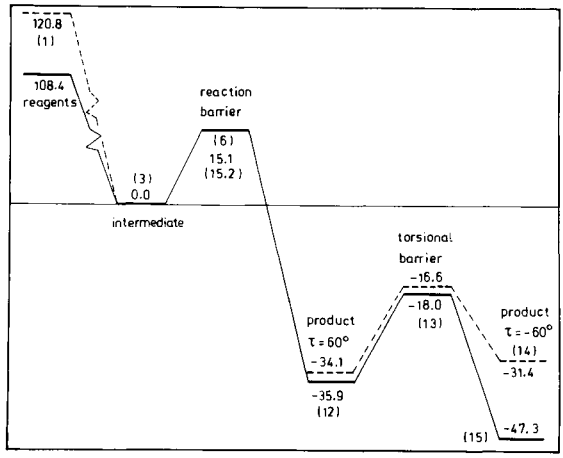

Fig. 8. 7s3p/3s (full lines) and STO-3G (dashed lines) minimum-energy reaction paths of β-lactam + OH^-. Energy in kcal mol^{-1}.

ANALYSIS OF THE MOLECULAR ORBITALS AND ATOMIC CHARGES

The analysis of the evolution of the highest molecular orbitals (HOMO's) and of the atomic charges during the reaction paths gives a possible interpretation of the similarities between the β-lactam + OH^- and CH_3O-β-lactam + OH^- reactions and of the differences between these reactions and the 3-cephem + OH^- one.

TABLE 3 Atomic Localizations of the First Two HOMO's

Reaction	Point	Second HOMO	First HOMO
β-lactam + OH^- and CH_3O-β-lactam + OH^-	substrate	O[a]	N_5, O_9
	intermediate	O_9, O	N_5, O_9
	barrier	N_5	N_5, O_9
	product	N_5, O	N_5, O
3-cephem + OH^-	substrate	C_3, S_1	N_5, C_3, S_1
	intermediate	O_9, O	N_5, C_3, O_9
	barrier	N_5, S_1	N_5, C_3
	product	N_5, S_1	N_5, C_3

a) O atom of the amide group or of the methoxy group for β-lactam and CH_3O-β-lactam respectively.

Indeed Table 3 shows that the CH_3O group does not influence the type and energy of the first HOMO which in both reactions of the monocyclic β-lactams is practically a N_5-O_9 or N_5-O lone-pair orbital. Additional results (Petrongolo and others, 1980c) show that the first HOMO's which are appreciably different are the sixth and seventh

ones which are lone-pair orbitals on the methoxy O atom. A similar result is obtained from the data of Table 4 which reports a comparison between the group charges of the β-lactam + OH^- and CH_3O-β-lactam + OH^- systems.

TABLE 4 7s3p/3s Group Charges for β-Lactam + OH^- and CH_3O-β-Lactam + OH^-

Point	C_8O_9OH a)	N_5H_5 a)	methoxy group
substrate	-0.96(-0.96)	-0.16(-0.16)	-0.17
intermediate	-0.57(-0.56)	-0.28(-0.27)	-0.27
barrier	-0.22(-0.21)	-0.56(-0.55)	-0.27
product	-0.37(-0.36)	-0.47(-0.47)	-0.26

a) β-lactam and CH_3O-β-lactam respectively.

On the other hand Table 3 shows that the first HOMO of 3-cephem + OH^- is localized on the enamine group, with S_1 and O_9 contributions in the substrate and intermediate, respectively, while the second and third HOMO's are mainly N_5-S_1 lone-pair orbitals. These results point out a double conjugation between the π orbitals of N_5 and $C_4 = C_3$ and between the lone-pair electrons of N_5 and S_1. Moreover the population analysis reported in Fig. 9 shows that the evolution of the 3-cephem + OH^- system from the intermediate to the barrier occurs together with a strong charge transfer from the C_8O_9OH group to the $N_5C_4C_3$ one which is greater than that of the other two reactions (compare Fig. 9 and Table 4). One may thus conclude that the enamine group of 3-cephem has a remarkable electron-withdrawing effect which is due to the

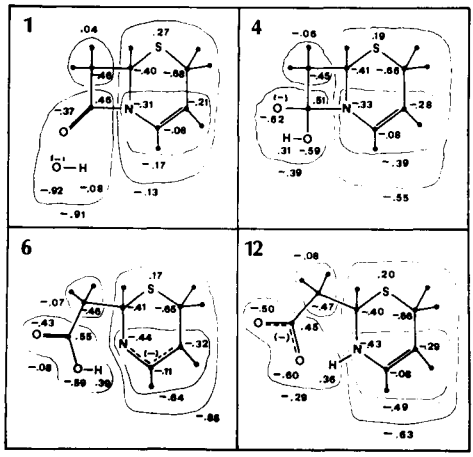

Fig. 9. 9s6p/7s3p/3s atomic and group charges of the 3-cephem + OH^- reaction.

enamine resonance and which strongly reduces the barrier height with respect to that of monocyclic β-lactams which are destabilized by an electron localization on N at the top of the barrier. The charge transfer is partially reversed in the following portion of the reaction path; nevertheless ∿50% of the negative charge of the product is localized on the enamine group.

CONCLUSIONS

The ab initio Hartree-Fock investigations give a detailed description of the mechanism of the base breaking of the endocyclic amide bond of β-lactams in some reactions which are suitable models of the central stage of the biological reaction between β-lactam antibiotics and enzymes. These calculations suggest a model of the activity of these antibiotics which can be compared with the experimental results.

Initial Attack of the Nucleophile

The strong stabilization of the intermediate with respect to the reagents is essentially due to the use of a strong ionic nucleophile with the consequent formation of a covalent bond with the amide carbon. A weaker ROH group is more suitable to describe the enzyme nucleophile; previous calculations on simpler systems (Schreiner and others, 1976; Kollman, 1977) give in this case a strong reduction of this stabilization (only non-covalent interactions are possible) in agreement with the experimental results. Useful information are however obtained by using OH^- and (more important) the following breaking of the amide bond should be less influenced by the nucleophile.

The calculations show that the OH^- attack to the β-face of the ring is unfavoured with respect to the α-face attack owing to the steric hindrance of the dihydrothiazine ring; of course this effect increases by considering larger 7β groups and ROH nucleophiles in accord with the experimental evidence for the α attack (Ghuysen and others, 1980). Likewise the calculated stabilization of the intermediate with sp^3 N_5 with respect to that with sp^2 N_5 can be correlated with the well known result (Sweet and Dahl, 1970) on the importance of the non-planarity of this atom for the antibiotic activity. However the N_5 hybridization of monocyclic β-lactams changes during the nucleophile attack, so that these compounds also can be active (Hashimoto and others, 1976) if electron-withdrawing groups are bound to N_5. Finally we note that the OH^- attack occurs in a similar way for all the considered reactions; this result is confirmed by Ghuysen and others (1980) which showed that the initial binding is not very selective.

Breaking of the Amide Bond

The methoxy substitution on the 7α position of the β-lactam ring does not influence this bond breaking, since the calculated reaction paths are very similar for β-lactam + OH^- and CH_3O-β-lactam + OH^-. This result corresponds to the kinetic one of Indelicato and Wilham (1974) which found nearly equal rate constants of base hydrolysis for 7-H- and 7α-CH_3O-cephalosporins. One may thus suggest that the methoxy group influences other steps not here considered as, for example, non-covalent interactions before the amide bond breaking.

On the other hand the dihydrothiazine ring of 3-cephem has a strong electronic and steric role on the reaction path, since the barrier height of 3-cephem + OH^- is about

one half of that of monocyclic β-lactams and also the energies of the products are remarkably different. This first result points out the lability of the amide bond of Δ^3-cephalosporins and it is mainly due to the π conjugation and electron-withdrawing effect of the enamine group; the second result shows the role of H-bond and steric interactions on the structure and energy of the product. Both the data of base hydrolysis of β-lactam antibiotics (Washkuhn and Robinson, 1971) and the importance of the enamine resonance in these compounds (Sweet and Dahl, 1970) confirm the theoretical study.

The analysis of the molecular orbitals points also out a conjugation between the lone-pair electrons of N_5 and S_1; however the role of the sulphur atom cannot be well estimated on the basis of the present calculations, since the effect of the enamine group is predominant.

The torsions of the β-lactam ring and of the COO^- group are important features of the reaction paths; moreover the product is stabilized by a strong hydrogen bond and by a spatial arrangement of the amide carbon which hinders a further nucleophile attack. These calculated conformational changes and stability of the product are rather similar to the corresponding results found by Ghuysen and others (1980) for antibiotic - enzyme complexes.

The present quantum-chemical calculations are in satisfactory agreement with some experimental data on the activity of β-lactam antibiotics. Of course further work is necessary to better correlate the theoretical and experimental results and to explain the activity of other antibiotics not here considered; we quote only a few possible future investigations: use of ROH nucleophiles, calculations on penam and 2-cephem, study of 3- and 7-substitutions, and effect of the aqueous solvent.

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Cyclic Analogues of Agonists as a Tool for Structure-Activity Relationships

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ABSTRACT

In order to obtain information on the steric requirements of the active sites of drug receptors, it is a matter of considerable interest to elucidate the steric und thermodynamic parameters that enable pharmacological compounds to initiate a response at a particular receptor. Due to the considerable flexibility of most of the drug molecules these aspects have to be studied indirectly by investigations of relationships between structure, stereochemistry and pharmacological activity of rigid or semirigid model compounds.

It is the aim of this review to discuss the advantages and also the disadvantages and limitations in using structurally rigid or semirigid analogues to deduce stereostructure-activity relationships. Examples are given how this concept has been applied in the field of muscarinic agents, morphinomimetics and tryptamines.

KEYWORDS

Stereostructure-activity relationships; cyclic analogues; bioisosterism; sulfonium analogues of parasympathomimetics; constrained tryptamines; constrained morphinomimetics; constrained muscarinic agents; active conformation.

INTRODUCTION

A large body of evidence has accumulated in the last decades that the mode of interaction of drugs with their receptors is largely a function of the chemical constitution and the mutual geometric disposition of the essential functional groups which interact with corresponding subsites in the receptor macromolecule. Since many drug receptors exhibit a high degree of stereoselectivity toward optical and geometrical isomers, it would not be unexpected for these receptors to possess conformational selectivity as well, and a great deal of effort has therefore been devoted to determining the active conformation of drug molecules (Lambrecht, 1976 a).

One approach to the study of conformational selectivity at drug receptors has been to determine the preferred conformation of the isolated molecule (theoretic

calculations), of the molecule in the crystal (X-ray crystallography), and of the molecule in solution (NMR spectroscopy) (Bergmann and Pullman, 1974; Casy, 1975). Although, studies of the conformational behaviour of drug molecules using the above mentioned physical methods are of considerable importance, the almost complete lack of knowledge about the conformational changes that occur in the drug and/or receptor during the drug-receptor interaction makes it difficult to deduce a stereostructure-activity relationship through conformational analysis alone.

Attempts to overcome this difficulty resulted in a unique experimental approach by which one is able to determine with a degree of certainty the active conformation of pharmacological compounds. This method involves the synthesis and testing of conformationally rigid or semirigid analogues of flexible drug molecules in which the possibilities of conformational variation are eliminated or greatly reduced.

METHODS OF CONTROLLING MOLECULAR GEOMETRY

Four different techniques of controlling the geometry of a drug molecule and of restricting rotations within the molecule may be used:

a) Making use of steric factors

The freedom to rotate may be limited, if the atoms forming a bond have large groups attached to them. The use of this approach to affect the stereochemistry of a flexible drug molecule, resulting in selectivity of action, is documented in a series of diphenhydramines (Ariens, 1977; Harms and Nauta, 1960).

b) Making use of multiple bonds

The relative positions of atoms attached directly to multiple bonds are fixed. In the case of double bonds, cis and trans isomers result. In relatively few cases the activities of olefinic cis/trans isomers have been reported, some examples are the tranquilizing thioxanthenes (Dunitz, Eser and Strikkler, 1964), the antidepressant zimelidine (Brown "and others", 1980; Coppen "and others", 1979; Ross and Renyi, 1977), the estrogenic stilbenes (Dodd "and others, 1938; Solmssen, 1945; Walton and Browlee, 1943; von Wessely, 1940), and cis- and trans-4-aminocrotonic acid as GABA analogues (Johnston "and others", 1975).

c) Making use of bioisosterism

There exist only a few reports in the literature in which the principles of bioisosterism were used to deduce stereostructure-activity relationships. One example of this approach is provided by pharmacological and stereochemical data comparing sulfur and selenium congeners of acetylcholine as cholinergic agonists (Aldrich, 1975; Chidichimo, Lelj and Russo, 1977; Makryiannis, Sullivan and Mautner, 1972; Mautner, 1974; Mautner, Dexter and Low, 1972; Partington, Feeney and Burgen, 1972; Pullman and Courriere, 1972; Webb and Mautner, 1966).

Recently we started using another approach in controlling the geometry of pharmacological compounds with the aid of bioisosterism, namely the substitution of the ammonium group of tertiary muscarinic agonists by the sulfonium group in order to prevent heteroatom pyramidal inversion. Some of our results are given in the section "Cyclic Acetylcholine Analogues".

d) Making use of cyclisation

Rigidity or semirigidity can also arise because parts of drug molecules are held in a ring. Of a set of cyclic analogues of a flexible open-chain drug, it is assumed that only those which fit the receptor will be active. From a comparison of the stereochemistry of the constrained analogues with possible conformations of the original drug one can make conclusions with respect to the conformation of the parent substance which reacts with the receptor, and the barriers to conformational alteration during drug-receptor interaction.

At the present time, the use of cyclic conformationally constrained analogues of flexible drug molecules to investigate the participation of conformational isomerism in drug activity appears to be a feasible approach, although it is not without pitfalls. It is difficult to devise cyclic drug molecules of a given conformation without also changing some other physicochemical properties of the prototype drug. Conformationally restricted analogues can be more lipophilic or stronger bases than the parent molecule, and their pharmacological activity can depend not only on the change in shape but also on the new physical properties conferred by cyclisation. There is another problem in relating conformational isomerism with biological activity using cyclic rigid or semirigid analogues. Rigidity may deny a molecule the opportunity of undergoing a necessary conformational change during its interaction with the receptor. Too rigid cyclic conformers may, therefore, be sometimes misleading as some degree of flexibility may possibly be needed for interaction with the receptor, if only for the sake of mutual adaptability (Pullman, 1974). The maximum precision of a stereostructure-activity relationship is determined by the precision of the biological data used in the study. Therefore, tests which prove that the drugs under investigation act direct via a common receptor are of special importance. In the case of agonists, the correlation of potency alone with geometrical factors, neglecting affinity and intrinsic activity of the compounds may sometimes give misleading results (Shefter, 1971).

In attempts to investigate the participation of conformational isomerism in ligand-receptor interaction with the aid of cyclic analogues, much attention has been focused in our laboratory on the receptors of acetylcholine.

CYCLIC ACETYLCHOLINE ANALOGUES

In the course of our studies on cyclic acetylcholine analogues we concentrated first on arecoline, the main alkaloid of areca catechu. Arecoline (I) can be considered to be a cyclic derivative of the reversed carboxy-analogue of acetylcholine (II) (Fig. 1).

The muscarinic activity of arecaidine esters depends largely on the structure of the side-chain, on hydrogenation of the double bond in the ring and on quaternization of the ring nitrogen atom (Gloge, Lüllmann and Mutschler, 1966; Mutschler and Hultzsch, 1973). From methyl ester to ethyl ester the affinity to the muscarinic receptor is increased whereas the affinity and intrinsic activity show a sharp fell in the case of the propyl ester. The effect of multiple bonds in the side-chain on the pharmacological activity is shown in Fig. 2. Compared to arecaidine propyl ester (III), the affinity as well as the intrinsic activity of the allyl ester (IV) increase. The arecaidine propargyl ester (V) is more active than the standard compound carbachol and displays full intrinsic activity. Furthermore, ester V was found to be a compound which is more potent, in terms of its muscarinic effect, than acetylcholine.

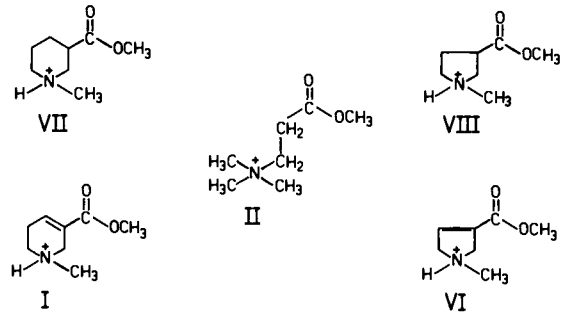


Fig. 1. Structural formulae of the reversed carboxy-analogue of acetylcholine (II), and of heterocyclic analogues.

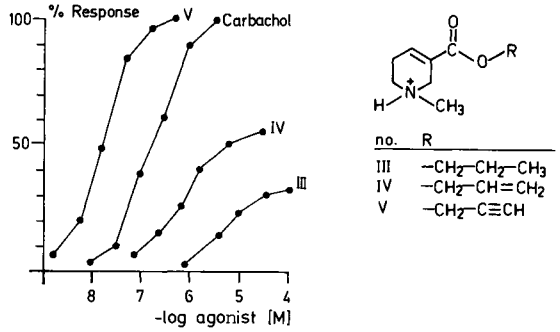


Fig. 2. Concentration-activity curves for carbachol and the indicated tertiary arecaidine esters on the isolated rat ileum.

In order to clarify whether the large differences in biological activity between compounds III - V are due to differences in the charge distribution, we calculated the electronic structure of these esters by the INDO MO method. There was hardly any difference in the charge distribution of the esters III - V. Therefore, it seems reasonable to assume that the potent muscarinic action of the propargyl ester V is due to an additional binding to the receptor of the triple bond.

Hydrogenation of the double bond in arecoline and in arecaidine ethyl ester causes a 25o - 1ooo-fold reduction in affinity to the muscarinic receptor. Probably this loss of muscarinic potency is due to a change in the molecular geometry. It could, however, not be excluded, that also the change in the electronic structure by loss of the conjugated system, could be responsible for the weaker muscarinic potency of the hydrogenated esters. The loss of potency by hydrogenation could be clarified experimentally by a comparison between arecoline and its corresponding analogue VI (Fig. 1).

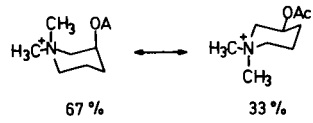
A change in the spatial orientation of the pharmacophoric groups is caused by hydrogenation of the pyrroline derivative VI, however, this change in molecular geometry is considerably smaller than that seen with arecoline, and accordingly the difference in muscarinic potency between the two 5-membered- ring analogues VI and VIII is smaller than that seen with arecoline and dihydroarecoline (VII) (Table 1). According to these investigations, the considerable loss of muscarinic potency which is found after hydrogenation of the arecaidine esters, is mainly due to a change in the steric properties and only to a lesser extent to a modification of the charge of the ester group (Hultzsch "and others", 1971).

TABLE 1 Muscarinic Activities of Heterocyclic Analogues of the Reversed Carboxy-Analogue of Acetylcholine[x]

no.	Equiactive molar ratios	VII/I	VIII/VI
Acetylcholine	1.00		
I	1o	25o	
VII	25oo		
VI	22		40
VIII	890		

[x]Guinea pig isolated ileum preparation.

Furthermore, about 15 years ago we started a stereostructure-activity study in an other series of cholinergic agents using (R,S)-3-acetoxy-N-methyl-piperidine methiodide (IX) as a chemical starting point (Fig. 3).



IX

Fig. 3. Structure and chair conformations of (S)-3-acetoxy-N-methyl-piperidine methiodide (IX).

Because it is well known that sometimes tertiary amines are actually more potent as muscarinic agonists than their quaternary derivatives, including arecoline (I) (Gloge, Lüllmann and Mutschler, 1966), and that the muscarinic receptor shows high stereoselectivity (Triggle, 1976), we synthesized in the first instance the tertiary (X) and quaternary (IX) individual enantiomers of 3-acetoxy-N-methyl-piperidine (Fig. 3 and 5) and tested these compounds for their muscarinic activity. The results of these experiments are collected in Table 2 (Höltje, Jensen and Lambrecht, 1979; Lambrecht 1976b, 1976d, 1980a; Lambrecht und Mutschler, 1974a, 1974b).

As shown in Table 2, all the semirigid cyclic analogues, based on the piperidine skeleton, are very weak muscarinic agonists. Excluding considerable differences in the electronic structures between acetylcholine (Pullman and Courriere, 1973) and the piperidines (Höltje, 1978), and remembering that muscarinic agonists and partial agonists activity is relatively independent of lipophilicity (Chang, Deth and Triggle, 1972), the low muscarinic potency of the semirigid piperidines

might only be seen as the result of stereochemical restrictions. Possibly too much energy is needed for the cyclic analogues to assume the required "muscarinic-essential" conformation during agonist-receptor interaction, possibly combined with direct steric hindrance; but these questions remained to be answered.

Table 2 Muscarinic Activities of Cyclic Acetylcholine Analogues[x]

no.	Compound	pD_2
	Acetylcholine	7.51
(R)-IX	(R)-3-Acetoxy-N-methyl-piperidine methiodide	3.4o[xx]
(S)-IX	(S)-3-Acetoxy-N-methyl-piperidine methiodide	3.92
(R)-X	(R)-3-Acetoxy-N-methyl-piperidine	2.99[xx]
(S)-X	(S)-3-Acetoxy-N-methyl-piperidine	3.68
(R)-XI	(R)-3-Acetoxy-quinuclidine methiodide	3.97
(S)-XI	(S)-3-Acetoxy-quinuclidine methiodide	3.56[xx]
(R)-XII	(R)-3-Acetoxy-quinuclidine	5.02
(S)-XII	(S)-3-Acetoxy-quinuclidine	6.10
(±)-XIII	(±)-cis-3-Acetoxy-1-methyl-thianium iodide	3.56
(+)-XIV	(+)-trans-3-Acetoxy-1-methyl-thianium iodide	6.50
(-)-XIV	(-)-trans-3-Acetoxy-1-methyl-thianium iodide	4.85

[x]Guinea pig isolated left atrium preparation, negative effects on the force of contraction. All experiments were performed in the presence of hexamethonium (0.15 mM), and di-isopropyl fluorophosphate (0.05 mM).
[xx]Partial agonists.

However, the results of Mashkovsky (1963) on the muscarinic activity of racemic 3-acetoxy-quinuclidine (XII) and its methiodide (XI) suggested an approach to our problem because these esters can be seen as rigid high energy boat form conformers of the semirigid piperidines, and the racemic tertiary ester was found to be a strong muscarinic agonist. Therefore, the individual tertiary and quaternary enantiomers of 3-acetoxy-quinuclidine (Fig. 4) were synthesized and tested for muscarinic activity (Lambrecht, 1976c, 1976d; Lambrecht and Mutschler, 1974a, 1974b).

no.	R
XI	CH_3
XII	H

Fig. 4. The absolute configuration of the (S)-enantiomers of 3-acetoxy-quinuclidines; R = H, CH_3 (Baker und Pauling, 1972; Belleau and Pauling, 1970; Meyerhöffer, 1972).

As shown in Table 2, the tertiary quinuclidine esters possess remarkable muscarinic potency, and the (S)-enantiomer may be used as a model for discussing fit to the receptor. The muscarinic potency of the corresponding quaternary compounds XI is much weaker, probably caused by steric hindrance due to the additional equatorial N-methyl group of the compounds.

From a comparison of the muscarinic potency of all the structures in the piperidine and the quinuclidine series (Table 2) with their stereochemical properties, and using the strong muscarinic potency of the tertiary (S)-3-acetoxy-quinuclidine for reference, we have postulated that the energetically unfavoured tertiary cis-(S)-boat form, shown in Fig. 5, with one axial nitrogen methyl group is a "muscarinic-essential" conformation of 3-acetoxy-piperidines. However, the important point is that the muscarinic receptor-agonist interaction seems not to be capable of overcoming the conformational energy barriers between boat and chair forms of the 6-membered heterocyclic saturated ring (Höltje, 1978).

The above mentioned experiments could, however, not exclude the possibility that also an energetically unfavoured chair conformation of the piperidine analogues with a proper spatial positioning of pharmacophoric groups is a "muscarinic-essential" conformation. Looking therefore for such a chair conformer, the stereochemistry of the 3-acetoxy-piperidines IX and X was investigated with ^{1}H NMR spectroscopy (Lambrecht, 1976d), and quantum chemical calculations using EHT and CNDO/2 MO methods (Höltje, 1978).

In the case of the 3-acetoxy-N-methyl-piperidine methiodides, it was found that the chair conformation with the axial acetoxy group is more stable than the one with the equatorial acetoxy group, but the energy difference is rather small (ΔG° = 1.8 kJ/mol) (Fig. 3).

In the case of the tertiary protonated 3-acetoxy-piperidines X four chair conformations should be taken into account (Fig. 5).

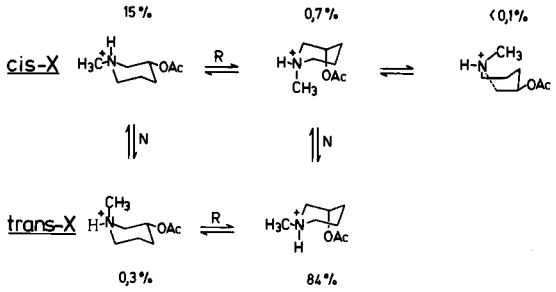


Fig. 5. Conformational isomerism in the tertiary protonated 3-acetoxy-N-methyl-piperidines. Only one enantiomer is shown. R = ring inversion; N = nitrogen pyramidal inversion via the free amines.

The chair conformation with the axial acetoxy group and the equatorial N-methyl-group is the most stable one, and the inverse chair with the equatorial acetoxy group and axial N-methyl group is the conformer with the highest energy. The difference in conformational energy between these two conformers is rather high, about 15 kJ/mol (Fig. 5).

However, comparing the proposed "muscarinic-essential" boat conformation (Fig. 5) and the highest energy chair conformer of the tertiary 3-acetoxy-N-methyl-piperidine (Fig. 5), it is obvious that these two structures have some relevant properties in common:
-the same absolute configuration at C3,
-the same spatial positioning of the acetoxy and the N-methyl group,
-trans arrangement in the N-C-C-O fragment (anticlinal/antiplanar), and
-both are high energy conformers.

The question here arising was: "How best to investigate the possibility of participation of the highest energy chair conformer in muscarinic activity of the semirigid 3-acetoxy-N-methyl-piperidine?" If this chair form is an active conformation with respect to the muscarinic receptor, then each alteration of the molecule which increases the population of this conformer should increase the muscarinic potency. This can be done by bioisosteric substitution of the ammonium group in the piperidines IX and X by the corresponding sulfonium group.

The sulfur atom in sulfonium salts may form a chiral center, and stereoisomers can be isolated, since the energy barrier for pyramidal inversion is substantialy higher (about 100 kJ/mol) than it is in the case of the corresponding ammonium compounds (about 38 kJ/mol) (Höltje, Jensen and Lambrecht, 1979). Therefore, four stable isomers of the sulfonium analogue of 3-acetoxy-N-methyl-piperidine may be isolated (Fig. 6) and tested pharmacologically.

Quantum chemical calculations (Höltje, 1978; Höltje, Jensen and Lambrecht, 1978, 1979), X-ray crystallography (Höltje, Jensen and Lambrecht, 1978, 1979; Jensen, 1979), and NMR spectroscopy (Eliel and Willer, 1977; Höltje, Jensen and Lambrecht, 1978, 1979; Lambrecht, 1977a, 1977b, 1978) have shown that the sulfonium analogues have another important advantage: As a result of the low value of the difference in conformational free energy for the sulfur methyl group (0.76 kJ/mol; in the piperidine series, this value amounts to 11 - 14 kJ/mol), the population of conformers having an axial methyl group in the onium center is much higher than in the piperidine series.

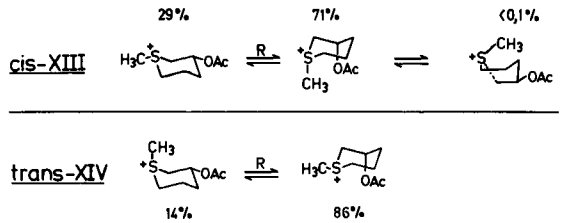


Fig. 6. Conformational isomerism in cis- and trans-sulfonium analogues of 3-acetoxy-N-methyl-piperidine. Only one enantiomer is shown. R = ring inversion.

In summary, the replacement of the ammonium group by the sulfonium group leads to a higher population (by a factor of about 100) of the possibly active chair conformation with axial heteroatom methyl group and equatorial acetoxy group in the trans series of the sulfonium analogues (Fig. 6). If the hypothesis is correct, we should measure a much higher muscarinic potency for one of the enantiomers of the trans-sulfonium compound XIV than for the corresponding ammonium

derivatives, and the cis-sulfonium ester XIII, respectively. This was in fact the case. Quantitative data for muscarinic activity of the sulfonium analogues are collected in Table 2 (Höltje, Jensen and Lambrecht, 1979; Lambrecht, 1977a, 1980a, 1980b). The following immediate observation can be made upon examination of the data in Table 2: The (+)-trans-sulfonium ester is more than two orders of magnitude more active as a muscarinic agonist than the ammonium compounds and the cis-sulfonium ester, respectively.

As a result of the very low population of boat and twist conformers (Höltje, 1978), one of the two chair conformers of the (+)-trans-sulfonium analogue, shown in Fig. 6, may be expected to represent a "muscarinic-essential" conformation of this agent. The difference in conformational free energy between the two chair forms amounts to only 4.5 kJ/mol. It thus seems highly possible that the conformer with the lower population of 14 % (Fig. 6) can be preferentially bound to the muscarinic receptor. The corresponding conformer of the tertiary 3-acetoxy-piperidine ester (Fig. 5) with an axial nitrogen methyl group and an equatorial acetoxy group is of much higher energy. In view of the low muscarinic potency of the tertiary piperidine esters X it might be suggested, that the active "muscarinic-essential" conformation is the one with the low population and that the energy barrier for these compounds is so high that it precludes a transformation of the low energy chair form with equatorial nitrogen methyl group and axial acetoxy group into the inverse high energy chair during agonist-receptor interaction.

The difference in conformational free energy for both chair conformers of the quaternary piperidine esters IX (Fig. 3) is as small as for the trans-sulfonium analogue, and the population of the conformer with an equatorial acetoxy group is rather high. The low muscarinic potency of these quaternary ammonium compounds may be seen as a result of direct steric hindrance due to the additional nitrogen methyl group of the compounds.

The transformation of the cis-sulfonium analogue XIII (Fig. 6) from any possible conformation into exactly the correct active chair conformation with axial sulfur methyl group and equatorial acetoxy group is not possible without cleaving bonds. On the other hand, this sulfonium ester can exist in the active boat conformation, shown in Fig. 4 and 5. But this boat form should be inaccessible during agonist-receptor interaction to some of the conformational modes of the molecule, due to the magnitude of the energy barrier separating the boat and chair conformations. These may be the reasons for its relatively low muscarinic potency.

We continued studying the stereostructure-activity in an additional series of cholinergic agents, namely the 4-acetoxy-piperidines and -thiacyclohexanes. The results obtained in these studies agree very well with the observations in the 3-acetoxy series (Höltje, Jensen and Lambrecht, 1978, 1979; Jensen, 1979; Lambrecht, 1978, 1979a, 1979b, 1979c).

CYCLIC FENTANYL ANALOGUES

Due to its high analgesic potency which is about 100-fold stronger than that of morphine, the 4-anilido-piperidine derivative fentanyl is particularly suitable for the investigation of structure-activity relationships in the field of morphinomimetics. Whereas the influence of various substituents in fentanyl derivatives on analgesic activity has already been investigated in depth (Casy "and others", 1969; Riley, Hale and Wilson 1973), only very little is known on the stereochemical requirements which have to be fulfilled by this class of drugs to ensure potent analgesia. Berger, Davidson and Langford (1977), Riley and Bagley

as well as our group (Klein, Back und Mutschler, 1974, 1975) were successful in synthesizing conformationally restricted analogues of fentanyl and testing them pharmacologically. The structural formulae of compounds synthesized in our laboratory are shown in Fig. 7.

By comparing the 3α- and 3ß-derivatives of the tropane series which were synthesized by Riley and Bagley (1979), it could be shown that the analgesic action depends more on the stereochemical properties than on other physico-chemical parameters, e.g. solubility, and that the pharmacophoric conformation of the 4-anilido-piperidine analgesics is identical with the thermodynamically preferred conformation: piperidine ring chair conformation with an equatorial 4-anilido moiety.

Berger, Davidson and Langford (1977) achieved the cyclisation of fentanyl by connecting the phenyl moiety with the piperidine ring to hexahydro-pyrido(4,3-b)indoles. These analogues, however, were inactive.

Both the above mentioned studies have one feature in common, namely that the propionyl moiety was not incorporated into a ring system. But this was done by our group resulting in compounds of the type XV, XVI and XVII (Fig. 7). However, these cyclic analogues of fentanyl showed no analgesic activity in the usual routine tests for analgesia. Also no morphine antagonism could be detected. The analgesic effect is apparently nullified by abolishing the ability of the aromatic ring to rotate around the N-C-axis and by fixation of the aromatic ring in the amide plane. It is interesting to note that, in contrast to fentanyl, a potent competitive H_1-antihistaminic effect is shown by these compounds. The cyclic analogue XVI ($R \triangleq OCH_3$) has a pA_2-value of about 9 on the guinea pig ileum. It is a compound which has an antihistaminic potency comparable to that of antihistamines in clinical use and which is 10^4-fold more potent than fentanyl.

The investigations so far described are good examples of how pharmacological properties are drastically changed by cyclisation reactions which apparently cause little change to the structure of the parent molecule. The importance of extending investigations on SAR beyond the properties of the parent molecule is further emphasized.

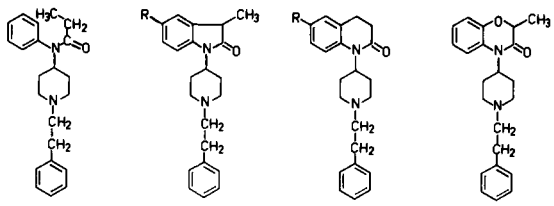
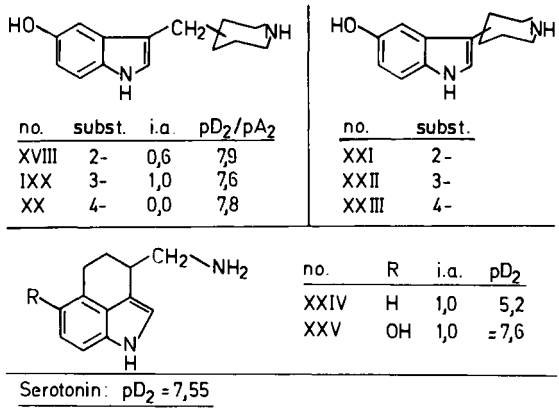


Fig. 7. Structural formulae of fentanyl and some of its restricted analogues.

CYCLIC SEROTONIN ANALOGUES

Literature on SAR of serotonin analogues is almost exclusively confined to investigations with derivatives with a flexible side-chain. We therefore synthesized cyclic serotonin derivatives in order to contribute to the clarification of the active conformation of serotonin. The compounds which were prepared and pharmaco-

logically investigated on the isolated gastric fundus of the rat are shown in Fig. 8 (Friderichs, Back and Mutschler, 1975a, 1975b).



no.	subst.	i.a.	pD_2/pA_2
XVIII	2-	0,6	7,9
IXX	3-	1,0	7,6
XX	4-	0,0	7,8

no.	subst.
XXI	2-
XXII	3-
XXIII	4-

no.	R	i.a.	pD_2
XXIV	H	1,0	5,2
XXV	OH	1,0	=7,6

Serotonin: $pD_2 = 7,55$

Fig. 8. Structural formulae of cyclic serotonin analogues.

Compound IXX possesses the same intrinsic activity and the same affinity as serotonin. These findings are noteworthy since other serotonin analogues known to date show less affinity than serotonin. In contrast, compound XVIII possesses only poor intrinsic activity and the corresponding derivative XX behaves like a competitive antagonist with the same affinity as serotonin.

Compounds with the piperidine ring directly connected to the indole moiety appeared to be even more interesting than the analogues XVIII - XX since the flexibility of these compounds is reduced even further. All the derivatives in this series (XXI - XXIII) showed an agonistic effect, but on repeating the experiments on the same fundus preparation, tachyphylaxis occured. Inhibition experiments with hexamethonium and atropine showed that part of the activity was due to an nicotininc effect and the rest of the activity probably due to a release of serotonin. Compounds XXI - XXIII are therefore no direct serotonin agonists.

Hence we looked for other cyclic analogues with a direct action on serotonin receptors. In this respect, compounds XXIV and XXV appeared to be particularly useful (Fig. 8). Pharmacological investigations with these derivatives (Klopp and Mutschler, unpublished results) on the gastric fundus of the rat showed that they are in fact direct serotonin agonists. The compound XXIV has a pD_2-value of 5.2 and an intrinsic activity of 1.0. The same data were found for tryptamine. Compound XXV, fully resembling serotonin, is a full agonist, but its pD_2-value could not be estimated accurately since the compound could not be isolated in a pure form. On the basis of the experiments which we performed, it is almost certain that the affinity of this agent does not differ to any substantial degree from that of serotonin.

The findings described above, show that our objective to produce rigid serotonin analogues with high potency was achieved with the derivatives XXIV and XXV. These investigations allow the assumption that the interaction of serotonin with the receptor takes place in the full trans conformation. It should be emphasized, that these conclusions only refer to serotonin receptors in the gastric fundus of the rat. The effects on serotonin receptors in the central nervous system were not examined, but the fundus strip appears to be a valid model for brain

receptors (Glennon and Gessner, 1979).

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Thromboxane A_2 and Atherosclerosis

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ABSTRACT

The discovery of thromboxane A_2 and prostacyclin has made clear the importance of the interrelationship between platelets and vascular wall in atherogenesis and thrombogenesis. It was confirmed in our laboratory that TXA_2 could contract endothelial cells to the extent of induction of abnormal vascular permeability and such was considered to be an important key mechanism in the initiation and progression of atherosclerosis. Clinical studies revealed the high levels of TXB_2 in plasma of patients with vascular disorders, as compared with levels in healthy controls and positive correlations between plasma levels of cyclic AMP and TXB_2 observed in healthy controls were not evident in these patients. The levels of plasma TXB_2 fluctuated in parallel with the clinical condition. Plasmal harmony reflects the exquisite interrelationship which can serve as a good target for the monitoring of these physical balances. Chemotherapy to right these unbalances will be essential for the prevention and treatment of atherosclerotic and thrombotic disorders.

KEYWORDS

Thromboxane B_2; prostacyclin; cyclic AMP; cyclic GMP; atherogenesis; endotheliopathy; plasmal harmony; phthalazinol; chemotherapy of atherosclerosis.

INTRODUCTION

A new discovery or invention often throws light on a theory or phenomenon which had once been disregarded or given little attention.

Since the first description by Rokitansky (1844) and the following theory of Duguid (1948),the thrombus theory of atherogenesis has been long discussed(Pfleiderer,1969; Schafer, Handin, 1979). However, in recent years the "cholesterol theory" has been given much more attention. The discovery of thromboxane A_2 in 1974 (Hamberg and others, 1974; Hamberg, Svensson, Samuelsson, 1975) and clarification of the physiological role together with the discovery of prostacyclin (PGI_2) (Moncada and others, 1976) contributed significantly to analyzation of platelets and vascular wall interrelationship (Needleman and others, 1976; Samuelsson and others, 1976; Moncada and others, 1977; Moncada, Korbut, 1978) and to a re-evaluation of the important roles of platelets and thrombosis in atherogenesis (Shimamoto and others, 1977a; Shimamoto,

1977b; Jørgensen, 1978; Nalbandian, Henry, 1978).

VASCULAR INJURY AND ATHEROGENICITY

Vascular injury is one of the most important key mechanisms in initiation and progression of atherosclerotic plaques (Shimamoto, 1963; Numano, 1977, 1978a, 1979a, 1979b). Our group have confirmed experimentally that in animals subjected to risk factors such as stress of painful stimuli, angiotensin II, epinephrine, cholesterol and cigarette smoke, there were edematous changes in the aortic wall which were characterized by acute plasmal infiltration into the aortic wall (Shimamoto, 1969; Numano and others, 1975a, 1978b). This infiltrate included large molecules such as VLDL and LDL due to the contraction and increased phagocytic activity of the endothelial cells (Shimamoto, Kobayashi, Numano, 1975). Furthermore, when such challenges were repeated in laboratory animals, there was a thickening of the intima in the aortic wall followed by formation of atheromatous lesions (Numano and others, 1975b). Robertson and Khairallah (1973) and Constantinides (1969, 1972) using radioactive lipoprotein or cholesterol also confirmed the infiltration of lipoprotein or cholesterol into the aortic wall through openings between the endothelial cells.

ENDOTHELIOPATHY

All these studies suggested the important function of endothelial cells in initiation and progression of atherosclerosis. Until recently, endothelial lines were thought to be only a boundary between the blood stream and the vessel wall and were regarded as a passive barrier. However, recent studies have made it clear that the endothelial line itself is an independent tissue synthesizing many hormones and substances such as histamine, collagen, factor III, plasminogen activator, tissue thromboplastin, heparin and having important physiological functions such as selective permeability, and the function of repelling platelets in the maintenance of the exquisite balance between the vessel wall and the blood stream (Schwartz, Ross, Lewis, 1978; Numano, 1980). Goldstein and Brown (1977) detected the LDL receptor in cultured fibroblasts and Bierman and Albers (1975) found the receptor in endothelial cells of the aorta, thereby indicating that the endothelial line is like a kind of control tower in the lipid metabolism in the vessel wall. Vane and Moncada (1980) also reported that 70% of the PGI_2 in the vessel wall was synthesized in the endothelial cells and that there was an antagonistic effect against platelet aggregation and vessel contraction by TXA_2. These findings clearly show the important roles of endothelial cells in biological and physiological functions in the vessel wall. Thus, atherosclerosis as characterized by lipid infiltration and accumulation which induces various tissue reactions and may be termed atherosclerosis as induced by "endotheliopathy" (Numano, 1979b, 1980).

THROMBOXANE A_2 IN VASCULAR INJURY

Thromboxane A_2 (TXA_2) induces a potent contraction of the vessel wall. Whether or not TXA_2 also contracts the endothelial cells has to be clarified in order to determine the possible occurrence of atherogenicity. Fig. 1A shows scanning electron microscopy of cultured rabbit endothelial cells. These cells were taken from the aorta of rabbits (Schwartz, 1978) and were cultivated for 10 passages. Cells proliferated in one layer as shown in Fig. 1A and Factor VIII was confirmed in these cells by using antiserum of Factor VIII to certify that these were indeed endothelial cells (Jaffe, Hoyer, Nachman, 1973).

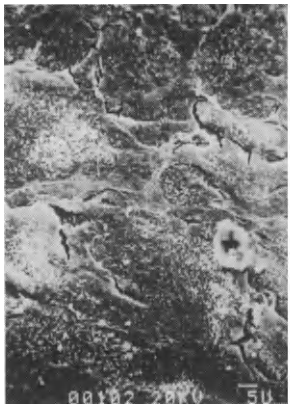

Fig. 1A

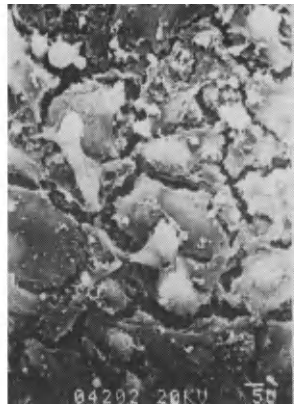

Fig. 1B

Scanning electron microscopy of cultured rabbit endothelial cells from the aorta (A) and following contractions induced by thromboxane A_2 (B)

A mixture of 50 μg of prostaglandin H_2 and 5 μg of microsomes had been mixed into the media in which 2000 pg/ml of thromboxane B_2 (TXB_2), a stable metabolite of TXA_2, was found to be synthesized 1 min after the mixing (Fig. 2). With this addition, these endothelial cells became swollen and ditch-like formations ran in all directions among the cells. Electron microscopic examination revealed folds, indentation and pinches in nuclei of these cells. Majno, Shea and Leventhal (1969) indicated that these changes occur in the nuclei with cell contraction. These preliminary studies also revealed a concomitant increase in the level of cyclic AMP in tissues and 6-keto-prostaglandin $F_{1\alpha}$, a metabolite of PGI_2, in the media, as shown in Fig. 2. Several workers have noted decreasing effects of cyclic AMP contents in the contracted smooth muscles with the application of TXA_2.

Our in vivo study which will be discussed in next chapter revealed decreased levels of cyclic AMP in endothelial cells injured by TXA_2. At present we cannot draw definite conclusions from these data. Such may reflect the temporary antagonistic response of endothelial cells to TXA_2 or the different response to the dose of TXA_2. However, this study does suggest that TXA_2 can induce contraction of the endothelial cells, that is, this compound may be an atherogenic factor in the induction of abnormal vascular permeability.

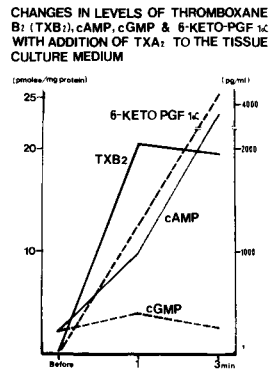

Fig. 2

Fig. 3 shows edematous changes in the aorta of rabbits in cases where TXA_2 was flushed through intraaortically (Numano, 1977). These edematous changes were confirmed in immunofluorescence studies using anti-β lipoprotein or γ-globulin antibody in which acute plasmal infiltration was produced. The content of cyclic AMP in the intima of the aorta of these so-treated rabbits decreased significantly, as compared with findings in healthy controls (Table 1). These results are identical with findings in the injured

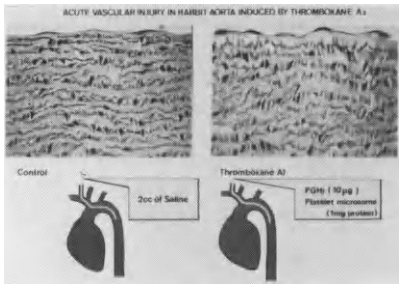

Fig. 3

Changes in the levels of cyclic nucleotides in intima and media of aortic wall of rabbits given one dose of thromboxane A_2 *

aortic wall	Groups	cyclic nucleotides (pmoles/mg protein) cAMP	cGMP
Intima	Placebo Control	1.12±0.09	0.23±0.06
	Thromboxane A_2	0.82±0.11	0.21±0.04
Media	Placebo Control	0.96±0.01	0.11±0.01
	Thromboxane A_2	1.32±0.12	0.14±0.01

P<0.01 : P<0.05 Placebo control vs thromboxane A_2
* the mixture of platelet microsome (5mg protein) and prostaglandin H_2 (50μg) was flushed into thoracic aorta through intraarterial catheter.

Table 1

intima of aorta of rabbits challenged with cholesterol, angiotensin II or epinephrine (Shimamoto, Kobayashi, Numano, 1975; Numano, and others, 1978b).

These studies strongly suggest that synthesized and released TXA_2 from platelets contracts the endothelial cells after which there is an infiltration of plasma including molecules of lipoprotein, inflammatory substances, various hormones, amines, growth factors (Ross, Glomset, 1976) and so on (Fig. 4).

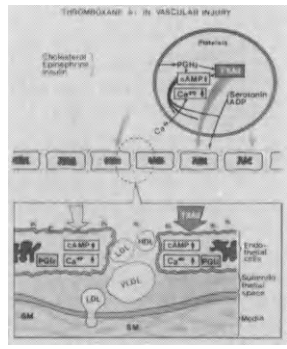

Fig. 4

THROMBOXANE A_2 IN THROMBUS FORMATION

Thrombus is an important factor in atherogenesis and produces fatal damage in patients with atherosclerotic disorders. Myocardial infarction, cerebral thrombosis or gangrene occurs with thrombus formation on atherosclerotic plaques to lead to a cessation in blood flow.

TXA_2 is a substance capable of accelerating platelet aggregation and thus initiating thrombus formation. Fig. 5A shows the human platelet aggregation as induced by collagen, With the progression of aggregation, TXB_2 is produced and there is a significant decrease in the levels of cyclic AMP in platelets. The injection of TXA_2 directly into the coronary artery of rabbits results very rapidly in a typical ST elevation followed by Q wave on the electrocardiogram. Histological examination revealed thrombus formation and evidence of myocardial infarction (Shimamoto and others, 1977a).

Fig. 6 shows changes in plasma TXB_2 levels in one patient. A 69 year old man in coma was hospitalized immediately after a cerebral vascular accident. The content of TXB_2 in the plasma at hospitalization was 420 pg/ml, a high level as compared with that in healthy persons. His clinical condition which remained unchanged in the first few hours gradually improved in response to the intensive care. The TXB_2 levels in the plasma as determined 4 hours after hospitalization showed higher levels than that at admission and then gradually decreased in parallel to the improvement of the clinical conditions. However, from the 5th hospital day on,

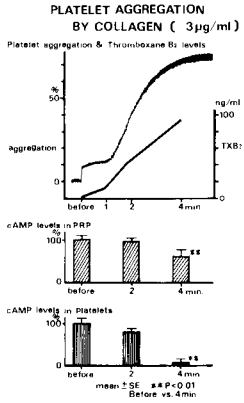

Fig. 5A

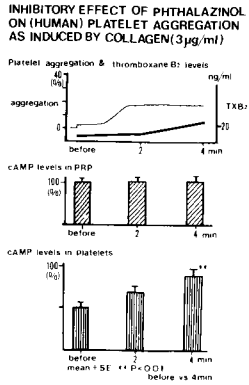

Fig. 5B

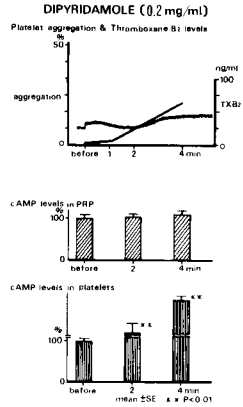

Fig. 5C

the TXB_2 levels again increased and he died on the 15th hospital day. Autopsy showed a cerebral thrombosis of the middle cerebral artery.

These data also strongly suggest the important roles of TXA_2 in thrombogenesis and associated clinical disorders.

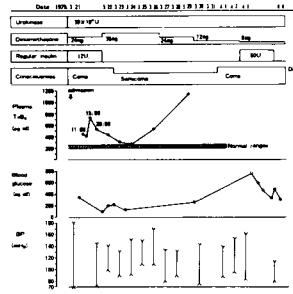

Fig. 6

PLASMA THROMBOXANE B_2 LEVELS

Table 2 shows the average levels of TXB_2 in plasma measured in 88 healthy young and old Japanese men and women. We designed a method to extract TXB_2 from a small amount of plasma samples using radioimmunoassay and I^{125}-TXB_2-Tyramide, a rapid and accurate method which can be used in any clinical laboratory (Koh and others, 1978; Sakanishi and others, 1979).

Although there was a gradual increase in the levels of TXB_2 with aging among men, the significance was not statistical. In women in their thirties and forties, TXB_2 levels were low as compared to these of a more advanced age. However, there were no statistical significant differences and the average level of healthy Japanese was 217 $\pm$ 9 pg/ml.
A positive correlation was seen between levels of TXB_2 and cyclic nucleotides in plasma of 30 healthy people as shown in Figs. 7A, B.
The plasma cyclic AMP is considered to mainly

Levels of thromboxane B_2 in plasma of healthy Japanese

Sex	Years	thromboxane B_2 (pg/ml)	
Men (N : 43)	10→	195.4±42.3	
	20→	222.4±24.4	
	30→	230.3±18.1	
	40→	252.2±30.5	
	50→	225.4±26.9	
	Total	228.2±12.6	216.9±8.6
Women (N : 45)	10→	262.2±38.0	
	20→	213.7±28.6	
	30→	191.1±21.5	
	40→	190.3±15.7	
	50→	220.8±31.2	
	Total	205.5±11.5	

Table 2

originate from the vessel wall and these data show the exquisite biological balance between blood and vessel walls.

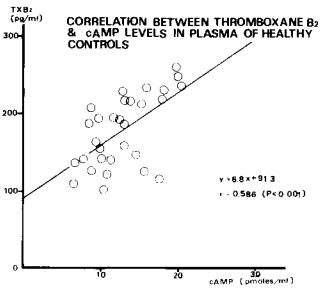

Fig. 7A

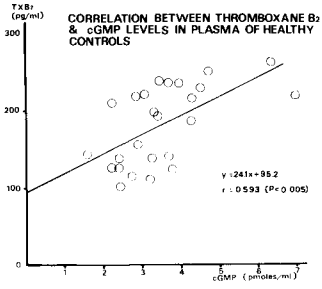

Fig. 7B

Table 3 summarizes plasma TXB_2 levels in patients with cardiovascular disorders. Statistically significant high levels of TXB_2 were confirmed in the plasma of patients with cerebral thrombosis, ischemic heart disease, Buerger's disease with ulcer, Takayasu disease and diabetes mellitus (Numano and others, 1979c). In such diseases, the thrombogenic factor is considered to be essential to the pathophysiological conditions, and correlations between TXB_2 and cyclic nucleotides levels in the plasma were negative (Figs. 8A, B).

Plasma thromboxane B_2 levels in vascular disorders

Disease	n	Plasma TXB_2 (pg/ml)
Cerebral Thrombosis	11	387.8±43.0
Cerebral Atherosclerosis	10	246.4±16.2**
Ischemic Heart Disease	41	290.0±16.7**
Atherosclerosis Obliterans	17	254.2±20.4
Buerger Disease (with ulcer)	41 15	245.0±14.0 306.8±53.0**
Takayasu Disease	53	254.8±18.5*
Diabetes Mellitus	7	427.3±56.3**
Healthy Controls	88	216.9±8.6

* P 0.05
** P 0.01 Controls vs. patients

Table 3

Our recent studies revealed that the plasma TXB_2 levels changed in parallel with the changes of clinical morbid conditions and we are now monitoring TXB_2 levels for the effective chemotherapy, rehabilitation and the prevention of reoccurrence.

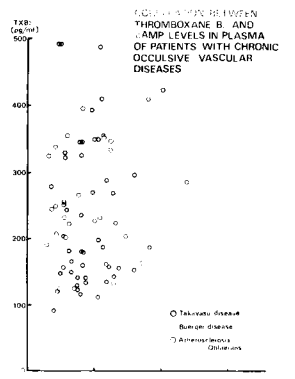

Fig. 8A

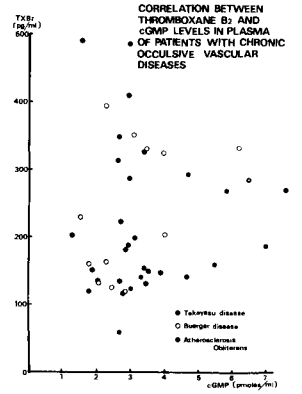

Fig. 8B

PHTHALAZINOL

Phthalazinol (EG626) is a phthalazin derivative with the chemical structure in Fig. 9. Pharmacological studies revealed the potent inhibitory effect of this compound on cyclic phosphodiesterase activity (Adachi, Numano, 1977). Experimental studies showed that this compound has an inhibitory effect on platelet aggregation and a favorable effect on the prevention and regression of experimentally induced atherosclerosis (Numano and others, 1976; Shimamoto, 1977b, 1977c; Numano, 1980).

Fig. 5B shows the inhibitory effect of phthalazinol on human platelet aggregation, as induced by collagen. The release of TXA_2 was significantly suppressed by phthalazinol, as compared with findings in platelet aggregation by collagen (Fig. 5A). The content of cyclic AMP in platelets was increased with the administration of phthalazinol and this compound was found to prevent the spiral strips of femoral, coronary and aortic wall from contracting with applications of thromboxane A_2. This antagonistic effect was dose dependent (Shimamoto and others, 1976).

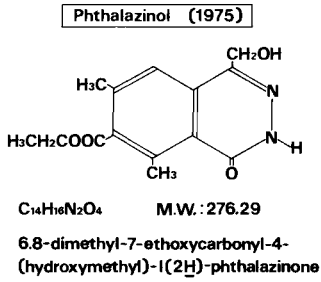

Fig. 9

Kuriyama's group (Suzuki and others, 1979; Kitamura and others, 1980) has also reported that phthalazinol relaxed the mesenteric and pulmonary arteries and attributed these effects to a possible suppression of the mobilization of Ca^{++}. Linder and Alksne (1978) also observed that phthalazinol prevented basilar arterial wall from contraction as induced by a perfusion of blood. Alksne and Branson (1979) reported that phthalazinol had a preventive effect against subarachnoid hemorrhage which led to arterial necrosis.

The antagonistic effect of prostacyclin from TXA_2 is considered to be achieved by the stimulation of adenyl cyclase in the membrane of platelets (Salzman, 1976; Haslam and others, 1978). Thus, with increased levels of cyclic AMP, the content of free calcium ion decreases and there is an inhibition of the release of TXA_2 from platelets. The inhibitory effect of phthalazinol for TXA_2 seems to follow the increase in levels of cyclic AMP.

Dipyridamole is an antiplatelet aggregability agent and has a cyclic AMP phosphodiesterase inhibitory effect (Mills, Smith, 1971; Moncada, Korbut, 1978) showed the same effects of increasing the content of cyclic AMP in platelets as was seen with phthalazinol (Fig. 5C). Dembińska-Kieć and Gryglewski (1977) reported that generation of prostacyclin in the vessel wall of rabbits reduced the progression of atherosclerosis and Neri Serneri and others (1979) observed the increasing effect of dipyridamole on the contents of prostacyclin in rabbit aorta in vitro. Tanaka, Harada and Katori (1979) have also reported that phthalazinol increased the content of prostacyclin in the vessel wall. At present it is unclear whether these relaxing effects are the direct effects of phthalazinol on prostacyclin metabolism and the possible increase in the levels of cyclic AMP is also a debatable factor. However, these biological properties will not only reduce platelet aggregability but also have favorable clinical effects on atherosclerosis.

Since 1976, clinical studies of phthalazinol have been done in neurological, angiological and cardiological fields and favorable results have been reported. Clinical improvement was seen also in patients with cerebellar ataxia and amyotrophic lateral sclerosis (Shimamoto and others, 1976). Brooks (1976) also confirmed the

increase in cyclic AMP levels in spinal fluid of patients with amyotrophic lateral sclerosis treated with phthalazinol and such was dose dependent.

Our own patients with peripheral and coronary vascular diseases improved remarkably while on this drug (Numano, 1977, 1978a). Motomiya, Sano and Shimamoto (1977) reported phthalazinol increases cardiac output and a possible enhancement of myocardial contractility in patients with ischemic heart disease.

As a representative case, a fifty-four year old man came to our clinic complaining of ulcers with severe ischemic pain and cyanosis on the right big toe and on the sole of his left foot (Fig. 10A). After 6 months on phthalazinol only, the ulcers were completely cured (Fig. 10B).

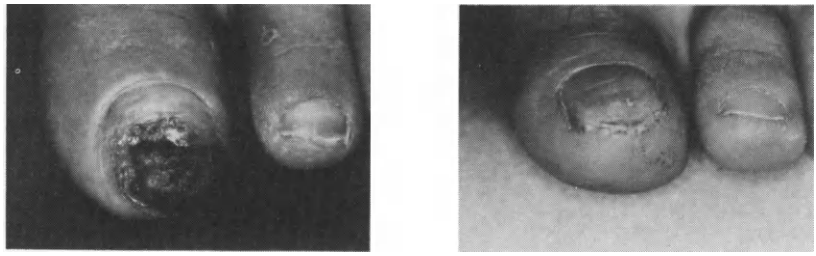

Fig. 10 Ulcer in a patient with Buerger's disease (E.Y. 54 yrs.)

A: before treatment B: after 6 months treatment with phthalazinol

Fig. 11 shows the changes in the plasma TXB_2 levels of this patient. The plasma levels of TXB_2 on his first visit were 438 pg/ml and while on phthalazinol there was a gradual decrease in these levels which paralleled the healing of ulcers. He went back to work after 5 months' hospitalization, and has had no recurrence. The monitoring plasma levels of TXB_2 is now a routine procedure in our clinic for patients with vascular related disorders.

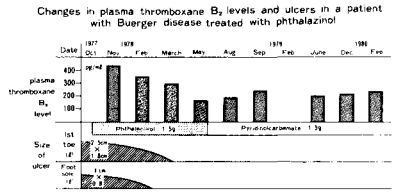

Fig. 11

CONCLUDING REMARKS

All these experimental and clinical data clearly demonstrate the important roles of thromboxane A_2 in atherogenesis and thrombogenesis and at the same time the close relationship between platelets and the vascular wall. Prostanoides and cyclic nucleotides play an important role in the control of this relationship. The balance of these local hormones is essential for the maintenance of health and an unbalance may indicate disorders. Thus, plasmal harmony reflects the exquisite interrelationship which could be a good target to monitor these physical balances. Chemotherapy to right these unbalances will be essential for the prevention and treatment of atherosclerotic thrombotic disorders.

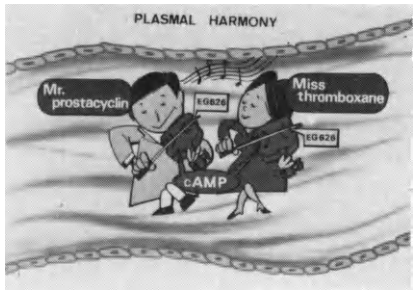

Fig. 12

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Biological Activities of Some Metabolites and Analogues of Prostacyclin

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ABSTRACT

The biological activities of prostacyclin metabolites and analogues on platelet aggregation, the cardiovascular system and the gastro-intestinal tract in various species are outlined. The 15-oxo and 13-14 dihydro metabolites of PGI_2 or 6-oxo-$PGF_{1\alpha}$ all had considerably reduced activity. Like prostacyclin, its analogues and PGI_3, the 13,14 dihydro products were not inactivated in the pulmonary circulation. The hepatic prostacyclin metabolite 6-oxo-PGE_1 was some 5-10 times less active than prostacyclin as an inhibitor of platelet aggregation and vasodepressor but, like prostacyclin, was a potent inhibitor of gastric acid secretion and gastric ulceration. The stable prostacyclin analogue, carbacyclin, was more potent as an anti-aggregating agent than the 5,6 dihydro analogue, 6β-PGI_1, and exhibited a biological profile comparable to prostacyclin. Both analogues also inhibited gastric acid secretion and gastro-intestinal ulceration induced by indomethacin. The 16-phenoxy prostacyclin analogue lacked platelet anti-aggregating activity and had little effect on the cardiovascular system, yet were extremely potent anti-secretory, anti-ulcer agents. Selectivity of biological action can thus be achieved within the prostacyclin series.

KEYWORDS

Prostacyclin; Prostaglandins; Metabolites; Analogues; Cardiovascular system; Platelet aggregation; Gastro-intestinal tract

INTRODUCTION

Prostacyclin [(5Z)-9-deoxy-6,9 α-epoxy-Δ^5-PGF_1] is a naturally-occurring potent but unstable vasodilator and inhibitor of platelet aggregation which is produced by the walls of blood vessels (Moncada, Gryglewski, Bunting and Vane, 1976). This bicyclic enol-ether derivative of the fatty acid precursor arachidonic acid is formed from the unstable endoperoxide intermediates by the action of an enzyme system, prostacyclin synthetase, located mainly in the vascular endothelial cells. The potent platelet actions and haemodynamic activities of prostacyclin (PGI_2) have implicated this prostanoid in the regulation of vascular tone and haemostasis (Moncada and Vane, 1979).

Prostacyclin has many potential clinical applications for the management of thromboembolic disorders and is valuable in preventing platelet aggregation during interaction with the artificial surfaces of extracorporeal circulatory systems. Prostacyclin thus improves haemocompatibiilty during charcoal haemoperfusion in dogs (Bunting and colleagues 1979) and man (Gimson and co-workers, 1980), whilst studies in dogs and man suggest that prostacyclin can be used as an alternative to heparin for haemodialysis (Woods and colleagues, 1978; Turney and co-workers, 1980). In cardio-pulmonary by-pass experiments in dogs using a bubble oxygenator, prostacyclin in combination with heparin

preserved both platelet number and function with minimal fibrinogen consumption and deposition on the arterial filters (Longmore and co-workers, 1979). Preservation of platelet number and function by prostacyclin during extracorporeal oxygenation with a membrane has likewise been demonstrated (Coppe, Wonders, Snider and Salzman, 1979).

Prostacyclin also has great clinical potential in the treatment of peripheral vascular disease, its local intra-arterial administration leading to alleviation of pain, regression of necrosis and healing of ulcers in cases of advanced arteriosclerosis obliterans in man (Szczeklik and co-workers, 1979).

Prostacyclin might also be useful in other conditions where excessive platelet aggregation is involved, such as thrombotic thrombocytopenic purpura, myocardial infarction, or stroke. Some of the complications of pre-eclampsia (Reumuzzi and others, 1980) and the platelet component of the rejection process during transplant surgery (Mundy, Bewick, Moncada and Vane, 1980) may also respond to prostacyclin therapy. These latter applications, however, are at the moment more speculative and further work is needed before a definite therapeutic role for prostacyclin can be assigned for such utilities.

In the present paper we outline the major biological activities of some metabolites of prostacyclin and some recently developed stable prostacyclin analogues.

Metabolism of Prostacyclin

Prostacyclin is unstable at physiological temperatures and pH, hydrolysing to the product 6-oxo-$PGF_{1\alpha}$ (Johnson and co-workers, 1976). The chemical half-life of prostacyclin at $37^{o}C$ at pH 7.4 is of the order of 3 min. However, *in vivo*, the biological activity of prostacyclin is often more short-lived. For example, the $t_{\frac{1}{2}}$ for the vasodepressor activity of prostacyclin (0.25 $\mu g\ kg^{-1}$) in a dose reducing systemic blood pressure in rats by 36 $\pm$ 1 mm Hg is only 0.46 $\pm$ 0.06 min, suggesting that the effects are limited by biological inactivation rather than solely by chemical breakdown.

Unlike the more classical prostaglandins of the E and F series, prostacyclin had comparable vasodepressor actions when injected by the intra-aortic or intravenous route in rats (Armstrong and co-workers, 1978) or dogs (Dusting and colleagues, 1978) suggesting that pulmonary degradation is not of primary importance in prostacyclin inactivation. This finding, coupled with the release of prostacyclin from the lung has led to the concept that prostacyclin is a circulating hormone (Gryglewski and co-workers, 1978; Moncada and co-workers, 1978). The failure of prostacyclin to be metabolised during passage through the lung indicates that it is not a substrate for the pulmonary-uptake system required for metabolism by the enzyme, 15-hydroxy-prostaglandin dehydrogenase (15-PGDH) in intact lung (Hawkins and others, 1978) since prostacyclin is a substrate for the enzyme itself *in vitro* (McGuire and Sun, 1978). In contrast, the breakdown product 6-oxo-$PGF_{1\alpha}$ is not a good substrate for this enzyme (McGuire and Sun, 1978). Prostacyclin is metabolised during passage through the peripheral circulation (Mullane, Moncada and Vane, 1979), liver (Dusting and others, 1978; Gerkins and others, 1978) and kidney (Wong and others, 1979) although the rate and nature of the metabolic processes are complex. Studies on the composition of the urinary metabolites of prostacyclin following its infusion in rats indicate that it can undergo the metabolic transformations described for the more classical prostaglandins. Thus products resulting from 15-dehydrogenation, Δ^{13} reductase, β-oxidation 19 and 20 hydroxylation and oxidation have been identified (Sun and co-workers, 1979). Likewise, 6-oxo-$PGF_{1\alpha}$ resulting from chemical breakdown of prostacyclin *in vivo* may further be metabolised, and Pace-Asciak and co-workers (1977) have demonstrated that a significant amount of 6-oxo-$PGF_{1\alpha}$ was excreted unchanged. Furthermore, studies in dogs have indicated a rapid elimination of both prostacyclin or 6-oxo-$PGF_{1\alpha}$ from the plasma following intravenous infusion in dogs (Salmon and co-workers, 1978).

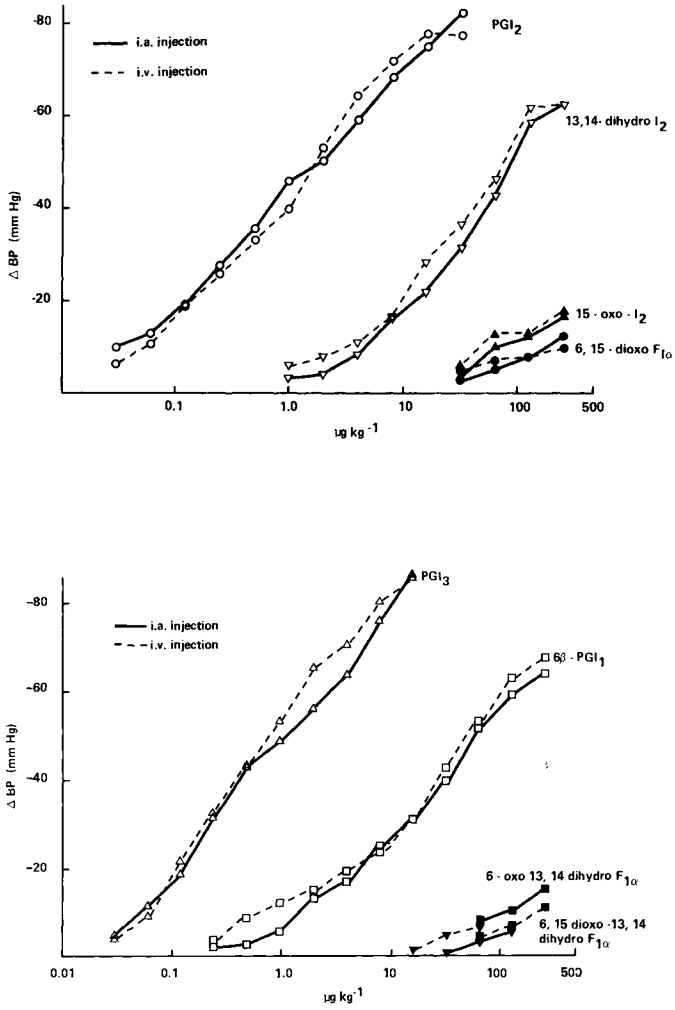

Fig. 1

Effects of prostacyclin, its metabolites and derivatives on systemic arterial blood pressure (BP) in the anaesthetised rat following bolus injection. Results shown as the fall in BP following intravenous or intra-aortic administration are the mean values for at least 4 experiments. Standard errors are omitted for clarity but were less than 5% of the mean.

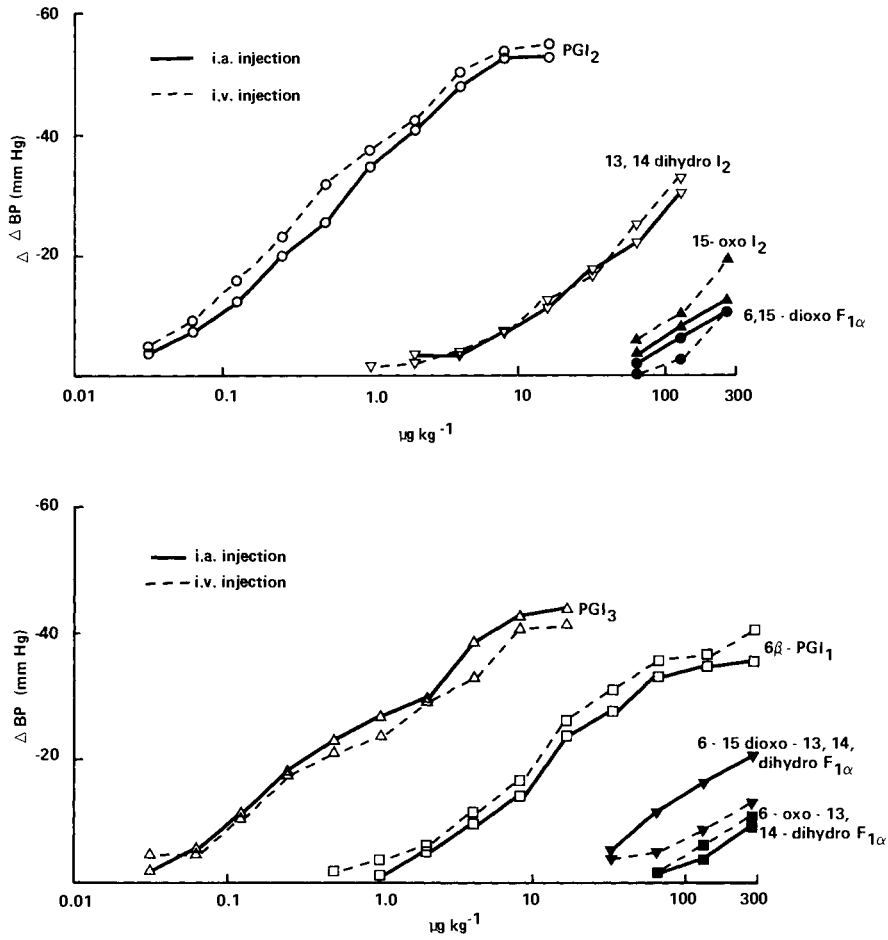

Fig. 2

Effects of prostacyclin, its metabolites and derivatives on systemic arterial blood pressure (BP) in the anaesthetised rabbit following bolus injection. Results shown as the fall in BP following inntravenous or intra-aortic administration are the mean values for at least 4 experiments. Standard errors are omitted for clarity but were less than 5% of the mean.

ACTIVITY OF PROSTACYCLIN METABOLITES

Cardiovascular actions

Anaesthesia was induced in male Wistar rats (250-300 g body weight) and male rabbits (2-2.5 kg) with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and maintained with suppliments (3 mg kg^{-1}). Arterial pressure was recorded from a cannulated femoral artery and heart rate derived by integrating the arterial pulse. Rectal temperature was maintained at 37°C by thermistor-controlled radiant heat. Each compound was injected into a femoral vein in a volume of 0.25 ml and flushed in with 0.25 ml of saline (0.9% w/v). Compounds were administered intra-arterially via catheters inserted retrogradely into the left carotid artery.

The various 15-oxo and 13-14 dihydro products of prostacyclin or 6-oxo-$PGF_{1\alpha}$ were all considerably less active than prostacyclin as vasodepressors in the anaesthetised rat (Fig. 1) and rabbit (Fig. 2). In both species, the primary product of the Δ^{13}-reductase enzyme 13,14 dihydro PGI_2 was more active than the product of the 15-PGOH enzyme, 15-oxo-PGI_2. The activity of the Δ^5-triene-prostacyclin, PGI_3, (Needleman and co-workers, 1970) which can be formed from the fatty acid substrate eicosapentaneoic acid (EPA) and the stable prostacyclin analogue PGI_1 (which cannot occur naturally) is also shown. In all cases, these prostacyclins had comparable vasoactive effects when injected by either the intravenous or intra-arterial route, indicating that little or no pulmonary inactivation of these compounds occurs.

The cardiovascular activity of the synthetic derivative 6,9 thioprostacyclin in the cat (Lefer and co-workers, 1979) and the coronary vasodilator activity of a 13,14 dehydroprostacyclin (Hyman and others, 1978) have also been described in detail.

Inhibition of platelet aggregation

Human blood was freshly collected into plastic vessels containing trisodium citrate (3.15%; 0.1 volume with 0.9 volume blood) and centrifuged (200g for 15 min) at room temperaturae. The platelet-rich plasma (PRP) was withdrawn into plastic containers and kept at room temperature. Inhibition of platelet aggregation was determined in a Born-type aggregometer as described previously (Whittle, Moncada and Vane, 1978) by incubating aliquots (0.5 ml) of the PRP for 1 min at 37°C with or without the inhibitor prior to addition of sufficient adenosine diphosphate (ADP; Sigma Chemical Co.) to just cause maximal aggregation. Dose-inhibition curves were constructed for each compound and the ID_{50} (dose causing 50% inhibition) was calculated as the dose required to reduce the control aggregation to 50% of its control amplitude.

As shown in Table 1, the metabolites of prostacyclin so far tested all were considerably less active than the parent compound as inhibitors of human platelet aggregation. As found for the cardiovascular actions, the 13,14 dihydro metabolite of prostacyclin was weakly active, whereas the 15-oxo metabolite was inactive up to 2 μg ml^{-1}. 6-oxo-$PGF_{1\alpha}$ was also weakly active whereas the 6,15-dioxo and 13,14 dihydro products had little activity. PGI_3, however, had potent anti-aggregating activity on human platelets.

It has been reported that the 20-methyl derivative of prostacyclin was slightly more potent than prostacyclin as a platelet anti-aggregating agent (van Oorp and co-workers, 1978). 20-methyl prostacyclin, like prostacyclin, prevents aspecifically-induced bronchoconstriction in asthmatic patients (Bianco and co-workers, 1978). The biological activity of some further unstable derivatives of prostacyclin (the 5,6 epimer, the 5,6 methylene and the 4,5 iso compounds) have also been reported by Crane and co-workers (1978) and 12-fluoroprostacyclins by Nicolaou and co-workers, (1978) but all showed reduced activity as platelet anti-aggregating agents.

TABLE 1

Inhibition of ADP-induced human platelet aggregation by prostacyclin, its metabolites and PGE_1 following 1 min incubation in vitro.

	ID_{50} (ng ml^{-1})	Relative Potency
Prostacyclin	0.4 ± 0.1	1
15-oxo-PGI_2	> 2000	< 0.0002
13,14 dihydro PGI_2	100	0.004
6-oxo-$PGF_{1\alpha}$	282 ± 37	0.0014
6,15-dioxo $PGF_{1\alpha}$	> 2000	< 0.0002
13,14 dihydro 6-oxo-$PGF_{1\alpha}$	> 2000	< 0.0002
13,14 dihydro-6, 15-dioxo $F_{1\alpha}$	> 2000	< 0.0002
6-oxo-PGE_1	6 ± 0.7	0.07
PGE_1	21 ± 2.5	0.019

Results, given as the ID_{50} value (dose causing 50% inhibition) and the relative potency to prostacyclin, are the mean ± S.E. mean from at least 4 experiments.

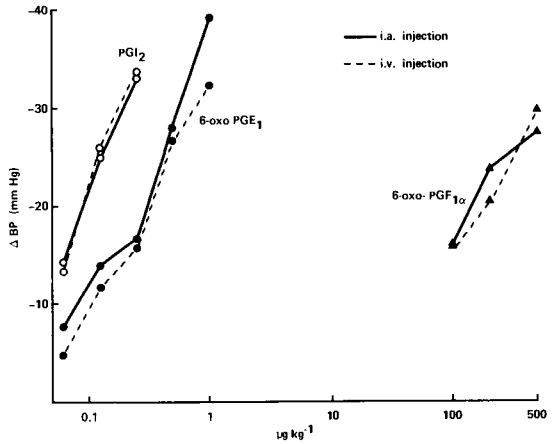

Fig. 3

Effects of prostacyclin, 6-oxo-PGE_1 and 6-oxo-$PGF_{1\alpha}$ on systemic arterial blood pressure (BP) in the anaesthetised rat following bolus intravenous or intra-arterial administration. Results, expressed as mean fall in BP, are from 4 experiments.

TABLE 2

Inhibition of ADP-induced human platelet aggregation by prostacyclin and its derivatives following 1 min incubation in vitro.

	ID_{50} (ng ml^{-1})	Relative Potency
PGI_2	0.4 ± 0.1	1
PGI_3	0.7 ± 0.2	0.57
6β-PGI_1	116 ± 20	0.0034
Carbacyclin	11 ± 3	0.036

Results, shown as ID_{50} values and Relative Potency to prostacyclin are shown as the mean ± S.E. mean from at least 4 experiments

Activity of 6-oxo-PGE$_1$

One of the more recently described metabolites of the hepatic metabolism of prostacyclin in vitro is 6-oxo-PGE$_1$. This chemically-stable product may arise from transformation of either prostacyclin itself or 6-oxo-PGF$_{1\alpha}$ via a 9-hydroxyprostaglandin dehdyrogenase pathway present in the liver, and may account for 7% of the total products (Wong and others, 1980). This compound has been shown to be a potent vasodilator (Quilley, Wong and McGiff, 1979) and has been claimed to be as potent inhibitor of platelet aggregation as prostacyclin itself (Wong, McGiff, Sun and Lee, 1979).

In our experiments in anaesthetised rats following bolus injections, 6-oxo-PGE$_1$ was some 4 times less active as prostacyclin as a vasodepressor (Fig. 3), and like prostacyclin was not inactivated following passage through the pulmonary circulation. Following intravenous infusion into anaesthetised rabbits 6-oxo-PGE$_1$ was some 5 times less active than prostacyclin as a vasodepressor agent (Fig. 4).

As an inhibitor of ADP-induced platlet aggregation in human plasma, 6-oxo-PGE$_1$ was only 0.08 times as active as prostacyclin (Fig. 5), with an ID_{50} of 6.1 $\pm$ 0.7 ng ml^{-1}. Our findings thus agree with the recent report by Miller and others (1980) that 6-oxo-PGE$_1$ was some 10-20 less active than prostacyclin as an anti-aggregating agent in vitro.

In further experiments, the inhibition of rabbit platelet aggregation ex vivo was determined in anaesthetised rabbits (Whittle, Moncada, Whiting and Vane, 1980). Male rabbits (2-2.5 kg body weight) were anaesthetized with sodium pentobarbitone and systemic arterial blood pressure (BP) was recorded from a cannula filled with heparinized saline (5 units/ml) in a femoral artery; no heparin was administered to the animal. Drugs were administered via a cannula in the jugular vein. Blood samples (3.0 ml) were slowly collected into a plastic syringe containing tri-sodium citrate (3.18%, 1 vol to 9 vol of blood) from a cannula inserted into the femoral vein, shaken gently and transferred to two Eppendorf plastic tubes (1.5 ml) and each was spun separately in a modified Eppendorf centrifuge for 2 sec (maximum centrifugal force, 10,000 g). The PRP from each tube was collected separately and 0.4 ml aliquots were transferred to the aggregometer and incubated at 37^{0}C for 1 min prior to addition of sufficient ADP (15 μM) to produce maximal aggregation. The time-interval between removal of blood samples and the transference of the PRP to the aggregometer was only 1.5 min.

Intravenous infusion of 6-oxo-PGE$_1$ inhibited ADP-induced aggregation in samples of plasma prepared by the 'rapid-spin' method with an ID_{50} of 2 μg kg^{-1}min^{-1}, being thus 10 times less active than prostacyclin (Fig. 4). This again agrees with in vivo studies on thrombus formation in dog coronary arteries where 6-oxo-PGE$_1$ was at least 10 times less active than prostacyclin (Miller and others, 1980). In our studies, this product produced a greater hypotensive response than prostacyclin at doses causing comparable degree of platelet aggregation, thus exhibiting a different profile of biological activity.

Prostacyclin, like the more classical prostaglandin, PGE$_2$, inhibits gastric acid secretion in both rat (Whittle, Boughton-Smith, Moncada and Vane, 1978a) and dog (Kauffman and co-workers, 1979). In the present study with 6-oxo-PGE$_1$ the gastric lumen of the urethane-anaesthetized rat was perfused with saline and systemic arterial BP recorded from a femoral artery. During submaximal rates of acid output stimulated by pentagastrin (0.5 μg kg^{-1}min^{-1}, i.v.) prostacyclin or 6-oxo-PGE$_1$ were infused for periods of 30 min and the degree of inhibition determined. The ID_{50} (dose causing 50% inhibition) was 0.2 μg kg^{-1}min^{-1}, i.v., for prostacyclin and 0.05 μg kg^{-1}min^{-1} i.v. for 6-oxo-PGE$_1$. Thus 6-oxo-PGE$_1$ was 4 times more active than the unstable prostacyclin following intravenous infusion as an anti-secretory agent in the rat.

Prostacyclin has also been shown to inhibit gastric erosions (Whittle and co-workers 1978a). The incidence and severity of gastric mucosal erosions formed in glandular mucosa 3 h

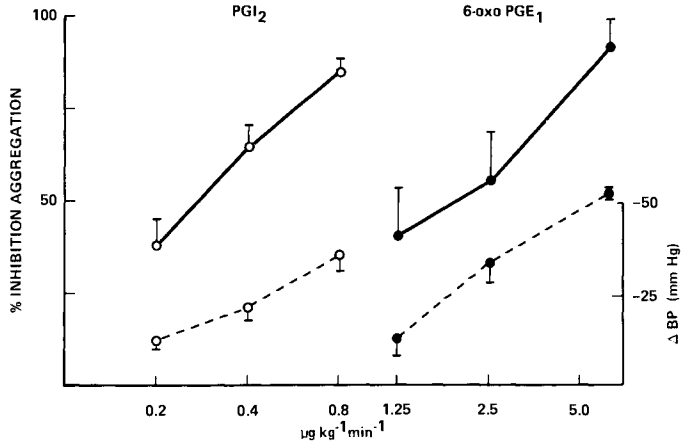

Fig. 4

Inhibition of ADP-induced platelet aggregation ex vivo and fall in systemic arterial blood pressure (BP) by intravenous infusion of prostacyclin and 6-oxo-PGE_1 in anaesthetised rabbits. Results expressed as % inhibition of platelet aggregation compared to the initial controls, and the change in BP, are shown as the mean ± s.e. mean of 4 experiments for each value.

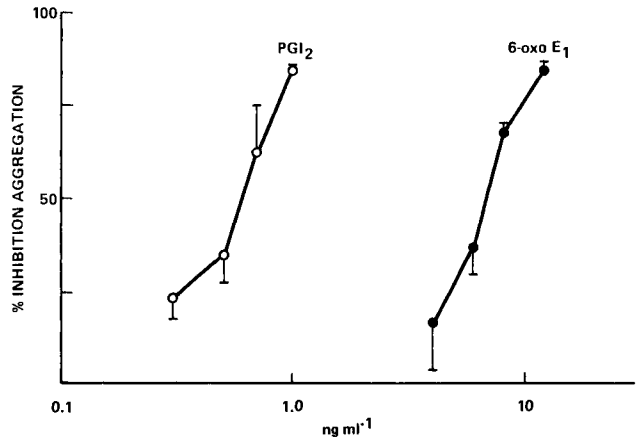

Fig. 5

Inhibition of ADP-induced platelet aggregation in human plasma in vitro following 1 min incubation with prostacyclin or 6-oxo-PGE_1. Results, expressed as % inhibition of control aggregation, are mean ± s.e. of 4 experiments.

after administration of indomethacin (20 mg kg^{-1}, s.c.) was assessed as described before (Whittle, 1976). Indomethacin (10 mg ml^{-1}) and the prostanoids were dissolved in 5% $NaHCO_3$ solution immediately before use. Like prostacyclin and PGE_2, inhibiton of gastric erosions was observed following administration of 6-oxo-PGE_1 (Table 4). However, in antiulcer doses, 6-oxo-PGE_1 produced mucoid diarrhoea in the rats with an 80% incidence of diarrhoea being observed with a dose of 125 µg kg^{-1} s.c., and a 100% incidence of diarrhoea with 250 µg kg^{-1} s.c. of 6-oxo-PGE_1. This contrasts with the lack of any such effects with antiulcer doses of prostacyclin or its analogues, (Whittle and Boughton-Smith, 1979) and suggests that 6-oxo-PGE_1 retains the potent effects of PGE_1 and PGE_2 in stimulating gastro-intestinal motility and diarrhoea. Thus, both prostacyclin and PGE's are potent vasodilators and are inhibitors of gastric acid secretion and ulceration whilst both prostacyclin and PGE_1 are considered to act at the same site on platelets to inhibit aggregation (Whittle, Moncada and Vane, 1978), yet prostacyclin and E-type prostaglandins have a markedly different profile of activity in stimulating intestinal motility and fluid secretion.

The contribution or relevance of 6-oxo-PGE_1 to the in vivo actions of prostacyclin have yet to be evaluated. However, because of the reported limited conversion of prostacyclin to this product in vitro, and its lower potency in the current studies, less than 1-2% of the biological activity of prostacyclin on platelets in vivo and on the cardiovascular system may be attributed to the formation of 6-oxo-PGE_1.

ACTIVITY OF STABLE PROSTACYCLIN ANALOGUES

6β-PGI_1

Because of the chemical instability of prostacyclin, it was considered of interest to develop chemically-stable analogues. One of the first described was a 5-6 dihydro analogue, 6β-PGI_1 (Johnson et al, 1977, 1979; Whittle, Boughton-Smith, Moncada and Vane, 1978b) whose structure is shown in Fig. 6. This analogue inhibited human platelet aggregation in vitro with an ID_{50} 116 ± 20 ng ml^{-1} (Table 2) being some 250 times less active than prostacyclin. As with prostacyclin, its vasodepressor activity was similar when administered by either intravenous or intra-arterial route in both rat and rabbit (Fig. 1 and 2). The epimer 6α-PGI_1 was less active on the cardiovascular parameters and platelet aggregation.

Studies were also carried out to investigate actions of 6β-PGI_1 on platelet aggregation ex vivo in the rabbit. As is shown in Table 2, 6β-PGI_1 was 80 times less active as prostacyclin as an anti-aggregating agent when infused intravenously and produced greater cardiovascular actions for a comparable degree of platelet inhibition. Thus the biological profile of 6β-PGI_1 differed from the parent, prostacyclin.

As with prostacyclin, 6β-PGI_1 inhibited gastric acid secretion in the anaesthetized rat (Whittle, Boughton-Smith, Moncada and Vane, 1978b) and conscious dog (Kauffman, and co-workers, 1979), being 8 times less active than prostacyclin when infused intravenously. Inhibition of gastric acid secretion in vitro with 6β-PGI_1 was also demonstrated, using the isolated lumen-perfused whole-stomach of the immature rat, where the prostanoids were added directly to the serosal solution (pH 7.6 at 37°C) and incubated for 30 min during stimulation of acid output with histamine (Whittle and co-workers, 1978a). Under these incubation conditions 6β-PGI_1 was the more active (Fig. 7), presumably reflecting the greater chemical stability of the analogue. Like prostacyclin, 6β-PGI_1 reduced 3 h indomethacin-induced gastric erosions with an ID_{50} of 250 µg kg^{-1}, s.c. compared to 350 µg kg^{-1}, s.c. for prostacyclin.

The biological activity of 5,6-dihydro prostacyclin analogues have also been described by other workers. Both Tonga and colleagues (1977) and Crane and co-workers (1978) have investigated the anti-aggregatory activity of both the 6α- and 6β- epimers on platelets. In a study on the isolated guinea-pig heart and bovine coronary artery strips, Schror (1979) has

TABLE 3

Effect of intravenous infusion of prostanoids on ADP-induced platelet aggregation ex vivo and systemic arterial blood pressure (BP) in the anaesthetised rabbit.

	Infusion $\mu g\ kg^{-1} min^{-1}$	Platelet Aggregation % inhibition	Δ BP (mm Hg)
PGI_2	0.25	42 ± 6	-24 ± 3
Carbacyclin	2.5	53 ± 8	-15 ± 3
$6\beta\text{-}PGI_1$	20	43 ± 13	-33 ± 10
PGE_1	2.5	7 ± 5	-24 ± 7
$6\text{-oxo-}PGE_1$	2.5	56 ± 13	-34 ± 6

Results are the mean ± s.e. mean of (5) experiments using doses of the prostacyclins close to their ID_{50} values.

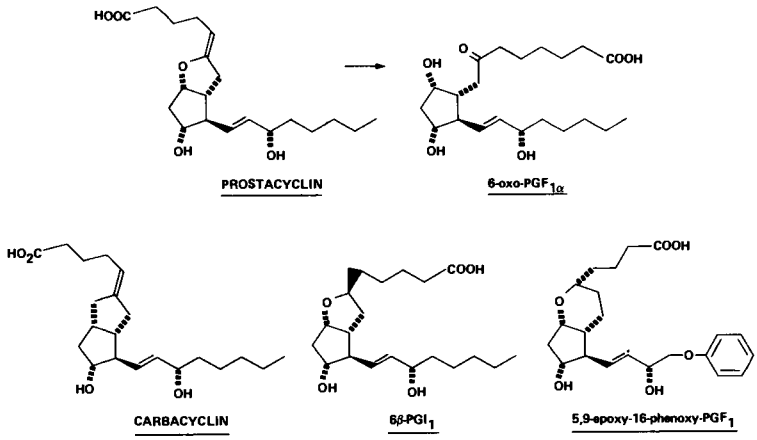

Fig. 6

Structure of prostacyclin and its stable analogues.

TABLE 4

Inhibition of pentagastrin-stimulated gastric acid secretion in the anaesthetised rat.

	($\mu g\ kg^{-1} min^{-1}$)	% Inhibition	Δ BP (mm Hg)
Prostacyclin	0.25	53 ± 10	-39 ± 4
	1.0	77 ± 6	-51 ± 7
PGI_3	0.1	63 ± 7	-18 ± 6
	0.4	88 ± 7	-22 ± 7
6-oxo-$PGF_{1\alpha}$	100	33 ± 2	-14 ± 2
	500	68 ± 7	-46 ± 9
6-oxo-PGE_1	0.02	32 ± 5	-3 ± 5
	0.1	68 ± 10	-13 ± 5
6β-PGI_1	2.0	27 ± 5	-9 ± 5
	10.0	77 ± 3	-21 ± 5
Carbacyclin	0.5	45 ± 7	-6 ± 1
	2.0	87 ± 7	-12 ± 3
5,9 epoxy 16-phenoxy	0.1	54 ± 3	-5 ± 3
	0.25	98 ± 2	+5 ± 2

Submaximal gastric acid secretion was induced by pentagastrin (0.5 $\mu g\ kg^{-1} min^{-1}$) and prostaglandins infused intravenously for 30 mins. The inhibition of acid output and concurrent fall in systemic arterial blood pressure is shown as mean ± s.e. mean of at least 4 experiments.

TABLE 5 Inhibition of indomethacin-induced rat gastric erosions by prostacyclin derivatives.

	($\mu g\ kg^{-1}$)	% Inhibition	(n)
Prostacyclin	250	25 ± 8	(18)
	500	68 ± 10	(18)
6-oxo-$PGF_{1\alpha}$	500	12 ± 9	(18)
6-oxo-PGE_1	125	62 ± 12	(5)
	250	78 ± 22	(5)
6β-PGI_1	125	23 ± 15	(30)
	250	50 ± 14	(10)
Carbacyclin	125	24 ± 14	(15)
	500	68 ± 12	(17)
5,9 epoxy 16-phenoxy	1	45 ± 5	(6)
	10	77 ± 7	(12)

Indomethacin (20 mg kg^{-1} s.c.) was injected immediately before the subcutaneous administration of the analogues and the erosion index assessed after 3 hr. Results, expressed as % inhibition of the control erosion index, are the mean ± s.e. mean of (n) values.

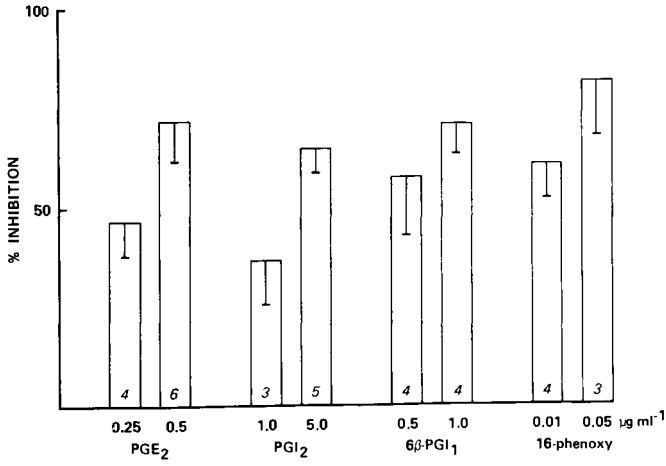

Fig. 7 Inhibition of histamine-stimulated (20 $\mu g\ ml^{-1}$) gastric acid output from the rat isolated whole-stomach by a 30 min incubation with prostanoids. Results, shown as % inhibition of control acid secretion are mean ± s.e. mean of (n) experiments for each value.

observed a divergent profile of activity of both epimers of PGI_1 with prostacyclin. Thus, these 5,6-dihydro analogues cannot be considered as close mimics of prostacyclin.

Carbacyclin

The synthesis of carbocyclic analogues of prostacyclin have recently been described (Morton, Bundy and Nishizawa, 1979; Nicolaou and co-workers, 1978). The chemically-stable prostacyclin analogue (5E) 6a-carba-prostaglandin I_2 (carbacyclin, Fig. 6) has proved to be a potent inhibitor of platelet aggregation in human plasma (Table 2). Carbacyclin was active against human platelet aggregation induced by ADP, arachidonic acid and collagen, and also inhibited platelet aggregation in plasma from a variety of species including dog and rabbit (Whittle, and co-workers, 1980; Aiken and Shebuski, 1980). As with prostacyclin the anti-aggregating action of carbacyclin was enhanced by the phosphodiesterase inhibitor, theophylline (Whittle and co-workers, 1980).

Carbacyclin was a potent inhibitor of *ex vivo* platelet aggregation when infused intravenously in the rabbit (Table 3) and dog, being one-tenth as active as prostacyclin. The inhibition of platelet aggregation reached plateau levels within 5 min of starting the intravenous infusion of carbacyclin and was maintained throughout the period of administration. In experiments where near-maximal anti-aggregating doses of carbacyclin were infused for 3 h, the degree of inhibition remained constant, showing no evidence for desensitization or tachyphylaxis of the platelets to this stable prostacyclin analogue *in vivo*. Likewise, Aiken and Shebuski (1980) has found this analogue to be effective in reducing thrombus formation in dog coronary arteries.

The anti-aggregating action of carbacyclin was, however, short-lived once the infusion was terminated. As with prostacyclin, inhibitory action on platelet aggregation *ex vivo* in the dog and rabbit was no longer significant 10 min after infusion. Thus, although carbacyclin is chemically stable at physiological temperatures and pH, , its similar duration of activity to prostacyclin suggests that in the rabbit and dog, both prostacyclin and carbacyclin are rapidly metabolised. Our studies in the rat indicate that carbacyclin is not inactivated during passage through the pulmonary circulation since the analogue had comparable vasodepressor activity when administered by intravenous or intra-arteiral injection. Like prostacyclin, carbacyclin may not be a substrate for the pulmonary-transport system required for metabolism by 15-PGDH enzyme in intact lung (Hawkins and others, 1978), yet may be metabolised readily in other organs such as the kidney or in vascular tissue (Sun and others, 1979).

Carbacyclin was active as a gastric antisecretory agent in the rat being 2.5 times less active than prostacyclin when infused intravenously. Furthermore, carbacyclin inhibited the acute 3h-development of rat gastric erosions with an ID_{50} of 290 $\mu g\ kg^{-1}$ s.c. and the more chronic 48h-development of intestinal lesions (ID_{50} 600 $\mu g\ kg^{-1}$ s.c.) induced by indomethacin (Whittle, Steel and Boughton-Smith, 1980).

16-phenoxy prostacyclin analogues

In an effort to develop prostacyclin analogues with greater tissue selectivity, especially as regards the gastric anti-secretory and anti-ulcer actions, a series of 16-phenoxy derivatives (Fig. 6) were synthetized (Johnson and others, 1979) and evaluated in several experimental models (Whittle and Boughton-Smith, 1979). Unlike 6β-PGI_1 and carbacyclin, these compounds failed to inhibit platelet aggregation *in vitro*. Furthermore, these compounds had only weak cardiovascular activity. Indeed, these compounds were found to induce a slight hypertension (of about 5 mm Hg) in antisecretory doses in rats (Whittle and Boughton-Smith, 1979) and dogs (Kauffman, Whittle, Aures and Grossman, 1980). The extremely potent antisecretory activity of one such compound, (5α) 5,9 epoxy-16-phenoxy PGF_1 has been demonstrated *in vivo* in the anaesthetised rat (Whittle and Boughton-Smith, 1979) and conscious dog (Kauffman and colleagues, 1980) and *in vitro* in the rat isolated

whole stomach (Whittle and Boughton-Smith, 1979). These compounds were also potent inhibitors of gastro-intestinal damage induced by indomethacin (Table 5) yet like prostacyclin had little activity in stimulating intestinal motility and diarrhoea. Thus these analogues offer both potency and selectivity of action within the prostacyclin analogue series.

Summary and Comments

It is apparent that the potent biological activities of prostacyclin are readily attenuated by even minor modifications in the chemical structure of this fatty acid. Prostacyclin, like other enol ethers undergoes facile hydrolysis in aqueous solutions to the product 6-oxo-$PGF_{1\alpha}$ which has markedly reduced biological activity. Metabolism of prostacyclin via either the 15-PGDH to 15-oxo-PGI_2 or the Δ^{13}-reductase enzymes to 13-14 dihydro PGI_2 *in vivo* likewise leads to greatly diminished activity, and the subsequent metabolites following consecutive attack by both enzymes are virtually devoid of biological activity. A recently described metabolite of prostacyclin formed by the liver *in vitro*, 6-oxo-PGE_1, retains potent actions as an inhibitor of platelet aggregation and vasodepressor, though is 5-10 times less active than prostacyclin. Since this product is only a minor metabolite and itself is likely to undergo further metabolic transformation *in vivo*, its formation may not enhance greatly the potency or prolong the duration of biological activity of prostacyclin. Indeed, since this metabolite is envisaged to arise as an oxidation product of 6-oxo-$PGF_{1\alpha}$ via a 9-hydroxy prostaglandin dehydrogenase pathway (Wong and co-workers, 1980), the weak biological activity of 6-oxo-$PGF_{1\alpha}$ administered *in vivo* could suggest that this proposed metabolic pathway to 6-oxo-PGE_1 may have limited importance *in vivo*. However, such a metabolic conversion is of considerable interest in the overall understanding of the regulation of prostanoid activity since it does represent an activation rather than the more usual de-activation process. Thus, the metabolite 6-oxo-PGE_1 is more potent as an anti-aggregating agent than its immediate precursor 6-oxo-$PGF_{1\alpha}$, and is also more potent than PGE_1 (Table 1).

The development of chemically-synthetized prostacyclin analogues have also demonstrated the strict requirement of the prostacyclin functional groups for the molecule to possess biological activity. The 5-6 dihydro analogue, 6β-PGI_1 is only a weak platelet anti-aggregating agent, although it did retain relatively more of the cardiovascular activity. Indeed, it appears that the binding sites on platelets for prostacyclin and its analogues are more stringent in their structural requirements than those on the vasculature.

Carbacyclin appears to be a very close mimic of prostacyclin with respect to its haemodynamic and platelet actions and is a chemically-stable but metabolically unstable analogue. Such analogues have clinical potential for the utilities previously proposed for prostacyclin itself. Furthermore, carbacyclin or similar compounds can serve as reference standards and help to elucidate biological actions and roles under conditions of temperature and pH which would limit the activity of prostacyclin itself. Selectivity of action within the prostacyclin series has clearly been achieved with the potent anti-ulcer 16-phenoxy analogues. Although these compounds are potent anti-secretory agents which like prostacyclin are devoid of stimulatory actions on gastro-intestinal motility, they fail to inhibit platelet aggregation and have slight hypertensive activity, rather than the potent hypotensive actions of prostacyclin. Thus these compounds no longer exhibit the two characteristic activities originally associated with prostacyclin, and their definition as prostacyclin analogues rests upon their chemical structure.

An explanation for this selective profile of biological activity may be sought in the proposed mechanisms underlying the various actions. Inhibition of platelet aggregation by prostacyclin and its analogues reflects stimulation of platelet cyclic AMP formation (Tateson, Moncada and Vane, 1977; Gorman, Bunting and Miller, 1977) whereas the inhibition of gastric acid secretion by prostacyclin and its analogues (Soll, 1979; Soll and Whittle, 1979) is considered to reflect the inhibition of cyclic AMP at the level of the acid

secretory parietal cell. Although prostacyclin may be capable of either activating or inhibiting adenylate cyclase depending on the tissue under scrutiny, analogues such as the 16-phenoxy derivative may selectively reduce cyclic AMP levels. Thus, it is clear that the rational development of highly tissue-selective prostacyclin analogues will depend on a understanding of the mechanisms involved with tissue response both at a level of receptor-binding site and the subsequent intracellular processes so initiated.

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The Role of Platelet Inhibitors in Degenerative Vascular Diseases

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This afternoon I would like to talk to you about a subject which has been of concern to mankind for many hundreds of years, namely, degenerative vascular diseases. This is not a single disease entity but is made up of several pathophysiological conditions which are associated with the cardiovascular system. Essentially, we are talking about hypertension, thrombosis, atherosclerosis and myocardial infarction. Each of these diseases is associated with an aberration, either direct of indirect, within the vascular bed. Moreover, unchecked hypertension and atherosclerosis inevitably lead to stroke and/or myocardial infarction. Although man has been afflicted with these problems since recorded time, I believe it is a truism that their occurrence has been most pronounced within the past century and a half. No doubt industrialization and increased urban demographic concentration and problems associated therewith have been to a considerable degree responsible for this. Moreover, the diet and quality of life in the developed nations of the world are also contributing factors.

Although the symposium this afternoon places special emphasis on platelets and their function, I believe it appropriate to focus briefly on some important concepts as related to hypertension which definitely relate to and have been instrumental in expanding our knowledge of platelets.

Nearly 25 million Americans suffer from hypertension. Complications commonly resulting from this insidious disease--renal failure, arteriosclerosis, cerebral hemorrhage and coronary artery disease--cause more than half of the adult deaths each year in the United States.

As recently as 30 years ago, the prevailing medical view was that elevated blood pressure was a protective compensatory adjustment to an ill-defined increase in the circulatory demands of the individual. Many even believed that lowering of the pressure would be harmful.

Until 1949 no effective drugs existed for controlling hypertension. But in a span of only 30 years since then, successive research gains have clearly shown that drug therapy can reduce blood pressure to normal range, much to the advantage--rather than detriment--of the patient.

A discovery which was fundamental to the eventual understanding of the medicinal control of blood pressure was made in 1946 by U. S. von Euler of the Karolinska Institute in Stockholm. He established that in mammals, including man, the sym-

pathetic neurotransmitter is norepinephrine rather than epinephrine. Epinephrine is the major neurohormone in the adrenal medulla in man, but norepinephrine is localized at the nerve endings.

This finding in turn led Ahlquist in 1948 to propose that sympathetic nerve stimuli are transmitted via two different types of receptors, designated as alpha (α) and beta (β) adrenergic receptors.

Thus, excess catecholamines, norepinephrine or epinephrine, impinging on the receptors could lead to increased cardiac output and an increase in peripheral resistance both of which effects cause an increase in blood pressure.

As a consequence research was directed toward substances influencing the action of the catecholamines on the α and β receptors. Specifically, extensive research was focused on drugs which would act as α or β-adrenergic receptor antagonists. An understanding of how these drugs acted at the molecular level was further elucidated by the discovery of cyclic AMP by Sutherland. He noted that the action of norepinephrine on the receptor led to activation of the enzyme adenylcyclase which converts ATP to cyclic AMP which functions as a second messenger. An increase in concentration of cyclic AMP brought about by excess neurotransmitter acting on the receptor in turn led to increased cardiac output and/or increased peripheral resistance.

I do not plan to go into the detailed history of the discovery and development of antihypertensive agents. However, in Table 1 are listed those important drugs and their mode of action.

The search for improved antihypertensive agents continues unabated following leads both from purely synthetic approaches as well as exploiting biochemical principles and substances of mammalian origin. An example of the biochemical approach is the development of the angiotension converting enzyme inhibitor captopril or Sq - 14,225. Although this substance is still undergoing extensive clinical trials, it is clear that is is an effective antihypertensive. Other modifications will surely be forthcoming from other laboratories.

Natural products have been a source of new drugs for many years. Although much of the work of medicinal chemists has been concentrated on substances indigenous to the plant kingdom (e.g. alkaloids) considerable attention has also been directed toward the study of natural products of mammalian origin.

By far one of the most challenging and promising areas of biological and clinical investigation in the past twenty years has been the prostaglandins. This indigenous substance was first identified over forty five years ago by von Euler (of norepinephrine fame) and also Goldblatt in accessory genital glands and human semen. The significance of this discovery is most profound in that it has influenced a great deal of research in elucidating the vital role of the essential fatty acids. This is now commonly known as the arachidonic acid cascade. The foremost investigators in this area of research have been Bergstrom, Samuelsson, Corey and Vane and Moncado of the Wellcome Research Laboratories.

TABLE 1

MODE OF ACTION OF ANTIHYPERTENSIVES

Drug	Mode of Action
Hydralazine	Vasodilation: Decrease in peripheral resistance by direct action on arteriolar walls and heart
Reserpine	Decrease in peripheral resistance by depletion of norepinephrine in granula
Guanethidine	Decrease in peripheral resistance by displacing norepinephrine in storage granulas
α-methyldopa	Decrease in peripheral resistance due to inhibition of norepinephrine synthesis and by central adrenergic depression
Clonidine	Decrease in peripheral resistance by interference with central adrenergic receptors
β-adrenergic blockers	Decrease in cardiac output and peripheral resistance
Prazocin	Decrease in peripheral resistance Inhibition of α_1-postsynaptic receptors

As already outlined by Moncado, one of the most interesting developments has been a better understanding in what might be called "platelet homeostasis". The brilliant research of Vane and co-workers, elegant in its simplicity, has shown that platelet homeostasis is maintained by a delicate balance in the two pathways from a common precursor. Thus, conversion of PGH_2 to thromboxane A_2 (TXA_2) causes a reduction in intraplatelet cyclic AMP and aggregation. Conversion of PGH_2 by the arterial wall to prostacyclin (PGI_2) causes platelet disaggregation and a rise in intraplatelet cyclic AMP. Thus, only at points of endothelian damage, where there is no prostacyclin synthetase, will collagen stimulated platelet aggregation occur. These findings by Vane have revolutionized current concepts in cardiovascular research.

The inhibition of platelet aggregation, even prior to an understanding of the detailed biochemical steps, has been considered an approach to prevention of myocardial infunction. In my presentation this afternoon I would like to focus primarily on a drug with which I was associated for several years while I was Executive Vice President and Director of Research of CIBA-Geigy, U.S.

In the mid-1960's Professor Mustard of McMaster University in Canada had noted that sulfinpyrazone was quite effective in extending platelet survival in patients with gout. In cooperation with the Medical Department of CIBA-Geigy Canada this finding was confirmed in several clinical trials.

Our discussion with Mustard involved the ability of sulfinpyrazone to interfere with the ability of platelets to stick and aggregate at the site of vascular injury and thus to prevent acute coronary deaths.

At about this time Dr. Christian Klimt, on behalf of the N.I.H., approached CIBA-Geigy with the proposition to carry out a long term multicenter trial with sulfinpyrazone. However, due to the excessive cost and extensive time schedule of his plan, it was my recommendation as Director of Research of CIBA-Geigy that we instead carry out the trial ourselves. It was my conviction that our Research and Development organization could carry out this trial more efficiently and at less cost.

Thus, in early 1975 we at CIBA-Geigy began to make plans to carry out a large clinical trial to determine whether or not sulfinpyrazone would reduce the risk of re-infarction in patients who had already suffered one event. We named this the Anturane Reinfarction Trial (A.R.T.).

We were also aware that considerable efforts were underway at the National Institutes of Health to carry out a large epidemological study with aspirin and with a combination of aspirin with dipyridamole.

However, the pharmacological actions of sulfinpyrazone suggested that it offered more advantages for evaluation than aspirin. Like aspirin, it inhibits the formation of thromboxane A_2 and collagen-induced platelet aggregation; it inhibits reentry ventricular arrhythmias arising from an acutely ischemic myocardium; it alters coronary vascular tone and increases collateral flow to the marginal zone of infarcted myocardium; and it is antithrombotic in several experimental models. Unlike aspirin, it is uricosuric in usual therapeutic doses; it protects the endothelium from cytotoxic agents; and it restores shortened platelet survival toward normal.

The Anturane Reinfarction Trial was undertaken in North America to compare the effects of sulfinpyrazone versus placebo on the rates of cardiac mortality in a double blind study among patients with a myocardial infarction followed for a minimum of one year and a maximum of two years. Patients with a recent myocardial

infarction were selected because of the high rate of mortality from an acute coronary event during the first year. As pre-calculated, approximately 1600 patients would be required to demonstrate a 50% reduction in mortality with statistical significance and this would require approximately 25 participating institutions.

The organization of this trial was unique in that it was a joint undertaking of the pharmaceutical industry and the academic community. Although the day to day conduct of the trial was the responsibility of the Pharmaceuticals Division of CIBA-Geigy, all aspects were supervised by a Policy Committee composed of academic personnel with considerable experience in the planning and overview of the various aspects of such a study.

The Anturane Reinfarction Trial extended over a 33 month period. This randomized, double blind, multicenter trial compared sulfinpyrazone (200 mg qid) and a placebo in the prevention of cardiac mortality and among 1558 patients followed for an average of 16 months, beginning 25 to 35 days after a documented myocardial infarction. The trial was completed in July, 1978.

All but one of the 106 deaths in the group were cardiac; 59 were sudden. The benefit of sulfinpyrazone was attributed entirely to a reduction in sudden death during the second through the seventh months following infarction, when there were 35 cardiac deaths in the placebo group and 17 in the sulfinpyrazone group; of these deaths, 24 in the placebo group and 6 in the sulfinpyrazone group were sudden cardiac deaths-giving a 74 percent reduction in the calculated mortality rate due to sulfinpyrazone.

These results are compared with the Aspirin Myocardial Infarction Study (AMIS) which included 4524 patients over a three year period. The results indicated that the regular administration of one gram of aspirin per day starting two months after infarction did not reduce three year mortality as compared to patients on placebo. However recent evidence suggests that the dose of aspirin used in this study might have been too high, since under these conditions not only is thromboxane A_2 inhibited, but the synthesis of prostacyclin is prevented. Thus, the balance between thromboxane and prostacyclin which is the control of platelet aggregability is seriously upset by high daily doses of aspirin.

The Persantine and Aspirin Reinfarction Study (PARIS) has not been published as yet but preliminary results appear more promising. One group of 810 patients in the study received 972 mg. of aspirin and 225 mg. of Persantine daily. Another 810 patients were given 972 mg. of aspirin and 406 received placebo.

Comparing just those receiving the combination therapy with the controls, the treatment group had 67% fewer coronary events--fatal or not--in the first four months of study, 65% fewer after eight months, and 56% fewer after 12 months, with the gap narrowing to around 33% after 24 months. Patients on aspirin alone had about 16% fewer heart episodes over this two-year period than those on placebo.

Interesting data emerge from analysis of the 400-odd patients entering the study within six months of their qualifying infarction. Those in this subgroup getting both drugs had a coronary mortality 51% below the control-group level after 24 months, while ones taking only aspirin had a rate 40% under the controls.

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Thromboxane-Dependent Platelet Aggregation: Role and Limitations

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ABSTRACT

Platelet aggregation is accompanied by the release of ADP and arachidonic acid (AA) derivatives, mainly thromboxane A_2 (TxA_2), which trigger further aggregation. ADP and TxA_2 were considered as final mediator of aggregation, and their release refered to as first and second pathway, respectively. In fact, inhibition of both pathways does not prevent platelet stimulation by important agents such as thrombin or collagen and a third pathway was postulated. Aggregation by AA is inhibited fully and unsurmountably by aspirin and is due to TxA_2 ; aggregation by ADP and by adrenaline is also inhibited by aspirin. We demonstrated that rabbit platelets stimulated by thrombin, collagen or the Ca^{2+} ionophore 23197 release a phospholipid known as "platelet-activating factor" (PAF-acether). PAF-acether is not released from ADP and AA-stimulated platelets, but only by those agents with which a third pathway is associated. PAF-acether itself activates platelets independently from first and second pathways. The elucidation of the role, mode of release and action of PAF-acether should provide a novel entry for the development of anti-aggregating and potentially anti-thrombotic agents.

KEYWORDS

Platelets, Platelet-activating factor, Thromboxane, Prostaglandins.

INTRODUCTION

Platelets are blood cells which adhere to the vessel wall as soon as a lesion is produced. Adhesion is followed by secretion of substances found within intraplatelet granules, the so-called dense bodies, particularly serotonin, adenosine diphosphate (ADP) and Ca^{++}, and by the release of arachidonic acid (AA). The latter is 5, 8, 11, 14-eicosatetraenoic acid, the precursor of prostaglandins (PG) E2, F2 and D2. It does not exist as such in the cells, but esterified to membrane phospholipids. ADP and AA induce platelet aggregation, which in vivo triggers the formation of the thrombus, needed for the conservation of life during hemmorhages, and unwanted when formed at the wrong time and at the wrong place, within the vessel, during thrombosis.

I. THREE PATHWAYS FOR PLATELET AGGREGATION

Since the work of Mustard's group (Packham et al., 1977 ; Kinlough-Rathbone et al, 1977), at least three different routes accounting for platelet aggregation are recognized. ADP mediates the first pathway, and the metabolites of AA (particularly thromboxane A2, TxA2), mediate the second. Platelet aggregation induced in vitro by a small concentration of thrombin is suppressed in the presence of an ADP scavenger, such as the combination of creatine phosphate and creatine phosphokinase, or by an inhibitor of the cyclooxygenase such as aspirin, which prevents the transformation of AA into TxA2. When higher amounts of thrombin are used, aggregation occurs irrespective of the ADP and AA inhibitors. Addition of ADP to rabbit platelets triggers only the first pathway, whereas ADP can trigger release of further ADP (from the dense bodies), when applied to citrated human blood. Activation of rabbit platelets by AA involves only the second pathway. Thrombin, collagen, and the Ca^{++} ionophore A23187 trigger the first and the second pathway, when applied at low concentrations, and a third one, when applied above a threshold . Collagen appears to act as low amounts of thrombin, according to Packham et al (1977), but according to Charo et al (1977), as well as to ourselves, it can trigger as well the third pathway. Differences may come from the use of different collagen preparations.

II. PLATELET-ACTIVATING FACTOR (PAF-ACETHER)

The hypothetical mediator of the third pathway of aggregation should show aggregating activity despite suppression of the first and of the second pathways. Such a substance was described in 1972 and named platelet-activating factor (Benveniste et al). Some of the features of its action on platelets were reported later (Henson, 1976 ; Cazenave et al., 1979). Platelet-activating factor was originally shown to be released from sensitized basophils upon immune or non immune challenge with different reagents. We know presently that its structure is that of a 1-0-alkyl-2-0-acetyl-sn-glyceryl-3-phosphorylcholine (Benveniste et al., 1979 ; Demopoulos et al., 1979). Thus, it is now termed PAF-acether. It can be released by cells other than basophils, such as macrophages (Mencia-Huerta and Benveniste, 1979), neutrophils (Lynch et al., 1979 ; Jouvin and Benveniste, in preparation). We have shown that PAF-acether is also released during platelet aggregation, and provided evidence that it might be the mediator for the third pathway of aggregation. Stringent criteria must be used to differentiate PAF-acether from other potential platelet aggregating agents present in biological fluids (Table I).

TABLE I : Criteria for characterization of PAF-acether

1. Aggregation of washed rabbit platelets in the presence of CP/CPK (inhibitor of the first pathway of platelet aggregation) and aspirin or indomethacin (inhibitors of the second pathway).
2. Rf of 0.35 in silicic acid thin-layer chromatography (chloroform : methanol : water, 70 : 35 : 7).
3. Retention time on silicic acid high pressure liquid chromatography identical to that of standard PAF-acether from hog leukocytes or synthetic PAF-acether. Inactivation after incubation with pancreas phospholipase A2 but not with lipase from Rhizopus arrhizus.

III. RELEASE OF PAF-ACETHER BY PLATELETS

Plasma-free rabbit platelets stimulated with the Ca^{++} ionophore A23187 release an aggregating substance which meets all known characteristics of standard hog leukocyte PAF-acether as defined in table I. The amount of PAF-acether formed is proportional to the concentrations of the ionophore applied, most of it being released to the supernatant (Chignard et al., 1979b). Stimulation of human platelets with the Ca^{++} ionophore also triggers the formation of PAF-acether (Chignard et al., 1979b).

IV. THIRD PATHWAY AND PAF-ACETHER

A putative mediator of the third pathway should comply with certain criteria, for its formation and activity. One important point is that PAF-acether induces aggregation in the presence of inhibitors of the 1st and of the 2nd pathways. PAF-acether also induces the release of the content of the platelet dense bodies : serotonin (Henson, 1976 ; Cazenave et al., 1979) or ATP (Vargaftig et al., 1980c) , thus sharing a similar property with the Ca^{++} ionophore and thrombin, even in the presence of indomethacin (Charo et al., 1977). Bivalent metal chelation, or stimulation of the intraplatelet cyclic AMP content with prostacyclin, also inhibits the aggregation by thrombin, and by PAF-acether as well (Vargaftig et al., 1980c). Another important point is that PAF-acether is formed not only when platelets are stimulated by the Ca^{++} ionophore, but also by thrombin, collagen, and carrageenan, a polysacharide which induces aspirin-resistant aggregation of rabbit and of human platelets (Vargaftig, 1977 ; Chignard et al., unpublished results obtained with F. Wal, INSERM). The amounts of PAF-acether thus formed are compatible with a role in inducing aggregation. In contrast, ADP and AA, which do not activate the 3rd pathway, fail to trigger the formation of PAF-acether.

V. PHARMACOLOGICAL CONTROL OF FORMATION OF PAF-ACETHER BY PLATELETS

As stated above, inhibitors of cyclooxygenase, such as aspirin or indomethacin, do not inhibit formation of PAF-acether. In case the latter has a role in thrombosis, this might explain the failure of aspirin as a therapeutic agent. In contrast, certain substances such as EDTA do inhibit formation of PAF-acether by platelets (Chignard et al., 1979 a and c), indicating a role for Ca^{++}. Incubation of the platelets with dibutyryl cyclic AMP also reduces the platelet synthesis of PAF-acether (Chignard et al., 1979a and c). Under similar conditions synthesis of TxA2 is reduced, an effect which was attributed to inhibition of phospholipase A2 (Minkes et al., 1977). The latter enzyme requires Ca^{++}, the storage of which within intraplatelet vesicles is increased by high cyclic AMP levels (Kaser-Glanzmann et al., 1978). Activation of phospholipase A2 is probably not only the trigger for formation of TxA2, by providing the arachidonate substrate (Bills et al., 1976 ; Rittenhouse-Simmon and Deykin, 1977), but may trigger as well the synthesis of PAF-acether. This possibility is stressed by the fact that bromophenacyl bromide, an inhibitor of platelet phospholipase A2 activity (Vargaftig et al., 1980), inhibits as well the formation of PAF-acether by platelets (Chignard et al., 1979 a and c).

If this hypothesis were confirmed, phospholipase A2 would be the hallmark for all sorts of platelet aggregation (Vargaftig et al., 1980a). Consequently, drugs which affect this enzyme, or its initial activation, might show as better antithrombotic agents as compared to inhibitors of cyclooxygenase.

It is paradoxical to note that phospholipase A2, which destroys PAF-acether, by hydrolysing the esterified acetate (see table I), would as well trigger its formation. The possible role of phospholipase C, which is present in platelets (Mauco et al., 1979 ; Billah et al., 1979) has not been studied as yet. Platelet phospholipase C activity is stimulated by thrombin, and this activation is inhibited by the increase of the platelet cyclic AMP content (Billah et al., 1979 ; Rittenhouse-Simmons, 1979).

CONCLUSION

It is likely that PAF-acether has an important role in platelet aggregation, but most informations were obtained on rabbit platelets, which are more sensitive to PAF-acether than human platelets, but much less than guinea-pig platelets. Presence of plasma reduces substantially the platelet sensitivity to PAF-acether, both in human and rabbit platelet-rich plasma. An in vivo effect is compatible with the pharmacological properties of PAF-acether, which kills rabbits when given i.v. at 30 ug/kg, and guinea-pigs at 0.2 ug/kg, from hypotension, bronchoconstriction and thrombocytopenia (Vargaftig et al., 1980c ; and Chignard et al., unpublished results).

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Pharmacological and Biochemical Studies on Benzodiazepines

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ABSTRACT

Experimental studies concerning the relationship between brain levels and pharmacological effects of benzodiazepines in rats are reported. Depending on the animal species, the route of administration and the time elapsed between drug administration and testing, a benzodiazepine may be active per se or through the formation of active metabolites. At equal pharmacological effects all the benzodiazepines tested displaced ^{3}H-diazepam specific binding to brain by about 50%.

KEYWORDS

Benzodiazepines kinetics and metabolism; brain levels; diazepam; clonazepam; oxazepam; temazepam; clobazam; camazepam; benzodiazepine receptors.

INTRODUCTION

Benzodiazepines represent a large class of compounds widely utilized in clinical practice for the treatment of anxiety, insomnia, convulsions and muscular rigidity (Greenblatt and Shader, 1974; Garattini and others, 1973). Pharmacological study of these therapeutic activities is generally based on three main tests: (i) anticonvulsant effect, usually against leptazol; (ii) muscle relaxant effect, usually by the rotarod test and (iii) anticonflict effect, a test based on the benzodiazepine's capacity to antagonize the action of suppressive stimuli on learned behaviour (Geller, 1964). In general there is good agreement between the antileptazol and the anticonflict activity of any one benzodiazepine, while the muscle relaxant effect is usually achieved at higher doses. This review aims to summarise this laboratory's experience in seeking correlations between the pharmacological activities of benzodiazepines and their concentrations in the brain in various animal species.

RELATIONSHIP BETWEEN ANTILEPTAZOL ACTIVITY AND BRAIN CONCENTRATIONS OF BENZODIAZEPINES

All the benzodiazepines prevent convulsions induced by leptazol in rats but their potency may differ considerably depending on the route of administration and the

time elapsed between administration and the leptazol challenge. However the brain levels achieved by an effective dose of benzodiazepines (ED_{50}) are consistent when the drug does not give rise to active metabolites.

Previous studies have shown for instance that in mice oxazepam protects 50% of the animals from leptazol convulsions when the brain levels are around 0.1 µg/g (Marcucci and others, 1972). This concentration remains constant independently of the dose used to obtain the anticonvulsant effect, the variables being represented by the route of administration and the time between oxazepam administration and leptazol (Garattini and others, 1973; Caccia and others, 1980a). Lorazepam, a derivative of oxazepam, appears to be more active because the ED_{50} against leptazol convulsions is lower than oxazepam, the effect being achieved at 0.02 µg/g (Marcucci and others, 1972). However the same antileptazol effect may be achieved at different brain concentrations in different animal species (Garattini and others, 1973; Marcucci and others, 1968).

Table 1 summarizes recent data obtained in rats by measuring brain levels of various benzodiazepines at the ED_{50} against leptazol. The measurement times and the routes of administration selected are such as to minimize the contribution of known active metabolites. There are large differences in the ED_{50} (about 10 times) for the various benzodiazepines but there is an even larger range for the active brain levels (about 180 times). In addition, the rank order for the ED_{50} is not the same as for brain levels; in some cases, for example diazepam, the brain concentration is higher than would be expected on the basis of the ED_{50}, while in other cases, e.g. 0-chloro-N-desmethyldiazepam, it is lower. These data indicate that for various benzodiazepines the in vivo disposition (absorption, metabolism, clearance, partition between plasma and brain) shows individual characteristics which are important in determining the ED_{50}.

TABLE 1 Brain Levels of Various Benzodiazepines in Rats at ED_{50} Against Leptazol

Drug	Time*	Route	ED_{50} (mg/kg 95% fiducial limits)	Brain level (µg/g ± S.E.)
Clonazepam	30	p.o.	1.12 (1.37-0.92)	0.019 ± 0.004
0-Chloro-DDZ	30	p.o.	1.48 (1.75-1.26)	0.020 ± 0.006
Niflurazepam	30	p.o.	1.30 (1.60-1.05)	0.040 ± 0.010
N^1-Desalkylflurazepam	30	p.o.	2.31 (3.07-1.73)	0.055 ± 0.010
Diazepam	15	i.p.	1.31 (2.20-0.80)	0.170 ± 0.020
Oxazepam	30	p.o.	6.74 (9.46-4.81)	0.260 ± 0.070
Clobazam	15	i.p.	8.70(12.30-6.10)	1.350 ± 0.220
CP 1414S	15	i.p.	11.40(14.20-8.90)	3.080 ± 0.039
Camazepam	5	i.v.	5.20 (7.00-3.80)	3.490 ± 0.520

*Time elapsed between the administration of the benzodiazepine and the challenge with leptazol (120 mg/kg i.p.).
DDZ = N-desmethyldiazepam
CP 1414S is 7-nitro-2-amino-5-phenyl-3H-1,5-benzodiazepine

As already mentioned, the animal species is an important variable in the potency of action of benzodiazepines. Table 2 shows for instance that the antileptazol activity of clobazam and of its metabolite N-desmethylclobazam is highest in mice followed by guinea pigs and by rats. It is interesting to note that in guinea pigs N-desmethylclobazam is probably very poorly absorbed on i.p. administration as indicated by the antileptazol activity when it is injected intravenously (Ballabio and others, 1980).

TABLE 2 Antileptazol Activity of Clobazam and N-Desmethylclobazam in Various Animal Species

Animal species	Minutes between benzodiazepine and leptazol	ED_{50} (mg/kg i.p. and 95% fiducial limits) Clobazam	N-desmethylclobazam
Mouse	30	1.37 (1.19 - 1.59)	12.20 (9.70 - 15.40)
	90	2.00 (1.68 - 2.32)	13.00 (11.37 - 17.38)
	180	4.93 (4.33 - 5.60)	14.69 (11.51 - 18.75)
Rat	30	10.28 (8.59 -12.30)	-
	90	32.73 (28.06-38.18)	>100.00
	180	100.00	
Guinea pig	30	2.72 (2.20 - 3.36)	-
	60	-	*6.97 (5.66 - 8.57)
	90	15.78 (4.91 - 6.80)	>100.00
	180	10.44 (8.87 -12.30)	-
	360	-	22.60 (17.42 - 29.30)

*Intravenous administration

IMPORTANCE OF METABOLITES IN THE ACTION OF BENZODIAZEPINES

Several benzodiazepines are extensively biotransformed *in vivo* giving rise to active metabolites. This represents a complicating factor in correlating pharmacological effects with pharmacokinetic parameters (Garattini and others, 1977). Previous studies have shown that the antileptazol activity of diazepam, for example, lasts longer in mice and guinea pigs than in rats (Marcucci and others, 1970), the reason being that in mice the effect is sustained by adequate brain levels of the metabolites N-desmethyldiazepam and oxazepam (Marcucci and others, 1970) and in guinea pigs by N-desmethyldiazepam (Marcucci and others, 1971) but in rats no active metabolites of diazepam accumulate in the brain (Marcucci and others, 1968). O-chloro-N-desmethyldiazepam is transformed in mice into the potent 3-hydroxylated derivative known as lorazepam. Clobazam is transformed into N-desmethylclobazam (Voltz and others, 1979) an active metabolite in mice (Caccia and others, 1980b; Fielding and Hoffmann, 1979). Temazepam is N-desmethylated to form oxazepam in rats and mice (Garattini and others, 1977).

Table 3 shows data concerning the ED_{50} of temazepam and oxazepam for anticonvulsant (antileptazol test) and muscle relaxant (rotarod test) activities, with the related brain levels. In the rat the effect of temazepam in both tests is partially due to the formation of oxazepam. In mice, when the time elapsed between temazepam and leptazol is 180 min, all the anticonvulsant effect of temazepam is due to the presence of oxazepam in the brain (0.22 μg/g oxazepam

when temazepam is given compared to 0.20 μg/g when oxazepam is given, both drugs being administered at the same effective doses).

The relationship between brain levels and the muscle relaxant activity is less clear although it seems that oxazepam formation plays a role in the effect of temazepam in rats and mice.

Table 4 summarizes other findings concerning diazepam, clobazam and CP1414S (7-nitro-2-amino-5-phenyl-3H-1,5-benzodiazepine) in the antileptazol, anticonflict and rotarod tests in rats. The efficacy in the antileptazol and anticonflict tests is similar for the three benzodiazepines, but the rotarod test requires a dose 2-3 times greater. The 1,5-benzodiazepines clobazam and CP 1414S appear less active than the 1,4 benzodiazepine prototype, diazepam. Brain levels are fairly proportional to the active doses, and the N-desmethyl metabolites (only for diazepam and clobazam) are present in a small percentage compared to the parent compounds.

TABLE 3 Pharmacological Effects and Brain Levels of Temazepam and Oxazepam

Drug	Route	Min*	Test	ED_{50}	Brain levels	
					Temazepam	Oxazepam
Rats						
Temazepam	i.v.	5	AL	0.18 (0.14-0.23)	0.24 ± 0.04	<0.025
Oxazepam	i.v.	5	AL	0.21 (0.17-0.27)	-	0.23 ± 0.04
Temazepam	p.o.	30	AL	4.12 (3.36-5.06)	0.15 ± 0.03	0.11 ± 0.03
Oxazepam	p.o.	30	AL	6.74 (4.81-9.46)	-	0.26 ± 0.07
Temazepam	p.o.	30	RR	6.39 (4.49-9.04)	0.32 ± 0.03	0.22 ± 0.04
Oxazepam	p.o.	30	RR	23.43(14.13-39.12)	-	0.71 ± 0.19
Temazepam	p.o.	180	AL	10.33 (8.02-13.30)	0.17 ± 0.06	0.08 ± 0.01
Oxazepam	p.o.	180	AL	10.30 (8.31-12.84)	-	0.27 ± 0.06
Temazepam	p.o.	180	RR	18.27(13.89-24.06)	0.23 ± 0.03	0.15 ± 0.05
Oxazepam	p.o.	180	RR	15.47(11.22-22.28)	-	0.52 ± 0.14
Mouse						
Temazepam	p.o.	30	AL	0.50 (0.40-0.63)	0.11 ± 0.03	0.07 ± 0.01
Oxazepam	p.o.	30	AL	0.52 (0.40-0.60)	-	0.17 ± 0.03
Temazepam	p.o.	180	AL	1.30 (1.00-1.69)	0.03 ± 0.01	0.22 ± 0.05
Oxazepam	p.o.	180	AL	1.58 (1.27-1.98)	-	0.20 ± 0.04
Temazepam	p.o.	30	RR	5.99 (5.68-6.33)	1.44 ± 0.20	0.92 ± 0.27
Oxazepam	p.o.	30	RR	6.39 (5.00-8.17)	-	2.00 ± 0.29

*Time elapsed between admistration of the drug and the test: (AL) antileptazol; (RR) rotarod.
ED_{50} are expressed as mg/kg (95% fiducial limits); brain levels as μg/g ± S.E.

TABLE 4 Pharmacological Effects and Brain Levels of Three Benzodiazepines in Rats

Drug	ED_{50} (mg/kg i.p.)		Brain level (µg/g)		ED_{50} (mg/kg i.p.)	Brain level (µg/g)	
	A	B	C	D	Rotarod	C	D
Diazepam	1.31	1.25	0.12	0.06	4.05	0.55	0.11
Clobazam	8.70	10.00	1.35	0.04	17.50	2.40	0.13
CP 1414S	11.40	10.00	3.08	-	41.48	11.88	-

A - Antileptazol test; B - minimal effective dose in conflict test (punished responses); C - Parent compound; D - N-desmethylated metabolite.

RELATIONSHIP BETWEEN BENZODIAZEPINE BRAIN LEVELS AND HIGH AFFINITY DRUG BINDING SITES

The recent discovery that the brain has high affinity binding sites (receptors) for benzodiazepines (Braestrup and Squires, 1977; Speth and others, 1978; Mohler and Okada, 1978) has stimulated much work. Our interest has been to correlate brain levels of benzodiazepines with the displacement of tracer doses of ^{3}H-diazepam bound to brain structures *in vivo*. Table 5 shows that the distribution of bound ^{3}H-diazepam is higher in the forebrain followed by hippocampus, brainstem and striatum and finally by cerebellum. Irrespective of the degree of bound ^{3}H-diazepam, an effective dose of diazepam (ED_{50} against leptazol given i.p.) displaces the labelled compound from high affinity binding sites preferentially from the forebrain, and a lower percentage in the other brain areas. The cerebellum seems to be the least sensitive, although this dosage of diazepam displaces ^{3}H-diazepam binding to about the same extent in all the brain areas considered (40%), a larger dose of diazepam (ED_{50} effective on rotarod) produces about the same displacement in the cerebellum (46%, unpublished results), but about 75% in the other brain regions. Other benzodiazepines (see Table 6) cause less displacement of ^{3}H-diazepam bound to the cerebellum than to the brain. The effect of oxazepam and CP 1414S in displacing ^{3}H-diazepam bound to cerebellum is negligible. It is noteworthy that all the benzodiazepines tested displace brain ^{3}H-diazepam *in vivo* by about 50% when utilized at the ED_{50} against leptazol regardless of the fact that the actual doses have a range of about 40 times. The fact that the

TABLE 5 Distribution of Bound ^{3}H-Diazepam in Various Parts of Rat Brain and Displacement by the ED_{50} (1.3 mg/kg i.p.) Against Leptazol of Diazepam Given 15 min Before.

Brain area	% ^{3}H-diazepam bound	Effect of diazepam % displacement
Forebrain	37.0	46
Hippocampus	25.0	32
Brainstem	25.0	32
Striatum	21.0	40
Cerebellum	16.0	40

^{3}H-diazepam binding *in vivo* was determined according to the method of Williamson and others (1978).

TABLE 6 Displacement of ^{3}H-Diazepam Bound to Rat Brain and Cerebellum by Several Benzodiazepines Given at the ED_{50} Against Leptazol

Benzodiazepine	mg/kg (time of pretreatment)	% displacement of ^{3}H-diazepam in vivo	
		Brain	Cerebellum
Diazepam	1.31 i.p. (15)	44.3	31.0
Clobazam	8.70 i.p. (30)	49.9	17.2
Camazepam	55.00 p.o. (30)	45.5	17.2
Temazepam	4.10 p.o. (30)	59.9	10.3
Oxazepam	6.70 p.o. (30)	50.2	2.0
CP 1414S	11.40 i.p. (15)	39.4	3.4

ED_{50} against leptazol displaces similar amounts of ^{3}H-diazepam from the brain but not from the cerebellum suggests that the high affinity binding sites for benzodiazepines located in the cerebellum are not an important factor in explaining the effect of these drugs against leptazol convulsions. As suggested by other authors (Klepner nd others, 1979) this may be related to different properties of the benzodiazepine receptors present in the cerebellum.

Table 7 is an attempt to correlate pharmacological effects, brain levels and high affinity binding sites of several benzodiazepines. All the values are expressed in μmoles or pmoles for easy comparison. In the case of clobazam it is evident that the anticonvulsant and anticonflict activities in rats are probably related much more to the presence of brain clobazam than N-desmethylclobazam, because the latter is less effective than the former in displacing ^{3}H-diazepam in vitro. In the case of diazepam it is more difficult to decide whether the parent compound or its metabolite is of importance for the anticonvulsant and anticonflict activities. In the case of camazepam it can probably be excluded that the parent compound is effective, the metabolites, temazepam and oxazepam, probably being responsible for its anticonvulsant effect.

Table 7 shows another interesting finding: in vitro as well as in vivo it takes about 3-4 times more benzodiazepine to increase the displacement of bound ^{3}H-diazepam from 50 to 75%. Furthermore an ED_{50} in the rotarod test corresponds to brain benzodiazepine levels equally active in displacing around 75% of the brain bound ^{3}H-diazepam.

CONCLUSIONS

Benzodiazepines are a large group of drugs which considerably differ in their pharmacological potencies, their kinetic parameters, their metabolic pathways and their capacities for high affinity binding in brain structures. These differences may be magnified or reduced depending on a number of variables which include animal species, route of administration and time elapsed between administration and testing.

Independently from the absolute doses and the brain levels reached, the ED_{50} for several benzodiazepines in terms of antileptazol and anticonflict effects corresponds to displacement of about 50% of the ^{3}H-diazepam bound in vivo to brain but not to the cerebellum.

TABLE 7 Correlation Between Pharmacological Activities and Brain High Affinity Binding for Several Benzodiazepines in Rats

Benzodiazepine	Test	ED_{50} µmol/kg	Brain level pmol/g	^{3}H-Diazepam % displacement in vivo	^{3}H-Diazepam IC_{50} in vitro (pmol/ml)
Diazepam	AL-CT	4.6 (i.p.)	D 596	45.7	6.4
			DD 185		5.7
Clobazam	AL-CT	29.0 (i.p.)	C 4266	50.2	260.0
			DC 210		580.0
Camazepam	AL	147.8 (p.o.)	CZ 699		950.0
			T 598	46.4	24.0
			OX 385		43.0
Temazepam	AL	13.7 (p.o.)	T 498	59.0	24.0
			OX 385		43.0
Oxazepam	AL	24.0 (p.o.)	OX 910	50.2	43.0
CP 1414S	AL-CT	29.1 (i.p.)	CP 7857	39.4	490.0
Diazepam	RR	14.2 (i.p.)	D 1754	75.3	25.0*
			DD 443		23.5*
CP 1414S	RR	106.7 (i.p.)	CP30306	66.9	2890.0*

*IC_{75}. Antileptazol test (AL); Conflict test (CT); Rotarod test (RR); Diazepam (D); N-desmethyldiazepam (DD); Clobazam (C); N-desmethylclobazam (DC); Camazepam (CZ); Temazepam (T); Oxazepam (OX); CP 1414S (CP).

The ED_{50} for the muscle relaxant effect of several benzodiazepines corresponds to displacement of about 75% of ^{3}H-diazepam bound to brain in vivo. The pharmacological effects of some benzodiazepines may be explained by the presence of metabolites in the brain rather than by the presence of the injected drug.

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Receptor Mapping

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ABSTRACT

The deduction of topographical features of the active sites of receptor macromolecules through the interpretation of structure-activity studies constitutes one approach to receptor mapping. This type of approach to mapping the central dopamine receptor is illustrated based on studies of various semi-rigid chiral antagonists related to butaclamol, of defined receptor site conformations. The receptor map developed comprises a planar catechol primary binding site of defined minimal dimensions composed of α and β regions, uniquely located with respect to a nitrogen atom location site and a complementary hydrogen bond donor site. In addition, a uniquely located lipophilic accessory binding site of defined minimal dimensions has been identified on the receptor macromolecule. The receptor map is depicted using a Cartesian coordinate system, or alternatively, by using the computer-based MMS-X molecular graphics system. The receptor map is used to rationalize the observed enantiospecificity of the dopamine receptor towards various chiral ligands.

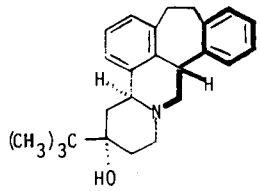
KEYWORDS

Receptor map; receptor model; pharmacophore; topography; dopamine receptor; enantiospecificity; antagonist; butaclamol; isobutaclamol; desoxybutaclamol; binding site; stereochemistry; crystal structure.

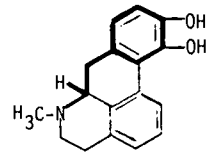
In recent years there has been a surge of interest in the question of ascertaining topographical features of drug receptors and several reviews have appeared (Gund; 1977, 1979, 1980; Marshall and co-workers, 1979). The most direct approach to achieving this goal is by the x-ray crystallographic investigation of drug receptor-ligand complexes. An outstanding example of this approach is the crystallographic study by Mathews and co-workers (1978) of the ternary complex between *Lactobacillus casei* dihydrofolate reductase, methotrexate, and reduced nicotinamide adenine dinucleotide phosphate. This study revealed the mode of binding of inhibitor and co-factor to the enzyme and suggests mechanistic details of the reduction process. Such information is of inestimable value in drug design and as pointed out by Mathews and co-workers (1978), can "provide an opportunity for rational design of a new class of DHFR inhibitors that would incorporate elements of both substrate and co-factor in a single molecule".

In principle it is a relatively straightforward task to obtain information on topo-

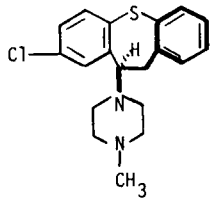
graphical features of drug receptors which are crystallizable enzymes. However, in the case of membrane-bound receptors such as those for the neurotransmitters, it has so far not been possible to obtain a crystalline receptor protein so that the direct observation of the receptor or receptor-ligand complex by crystallographic techniques is not possible.



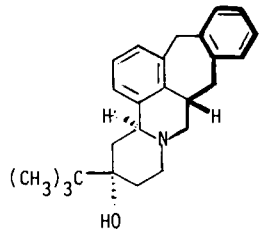
I. (+)-Butaclamol



II. (-)-Apomorphine



III. (+)-Octoclothepin



IV. (+)-Isobutaclamol

Faced with this situation the medicinal chemist must utilize an indirect approach, receptor mapping, to deduce topographical features of membrane-bound receptors. Receptor mapping is based on the premise that topographical features of a receptor are complementary to those of ligands with which it interacts. A detailed analysis of the molecular architecture of high-affinity, chiral ligands of defined receptor-site conformations can be interpreted in terms of topographical features of the recognition site on the receptor macromolecule.

This report describes studies aimed at mapping topographical features of the receptor(s) for the central neurotransmitter dopamine. These studies have evolved from a program which has resulted in the development of butaclamol (Bruderlein, Humber and Voith, 1975), a clinically-active antipsychotic agent (Clark and coworkers, 1977).

Butaclamol exerts its effects by blockade of central dopamine receptors (DAR), and is unique in that it was the first DAR antagonist wherein all the activity was due to one enantiomer (Voith and Cummings, 1976; Humber, Bruderlein and Voith, 1975). It was shown that (+)-butaclamol, I, was a potent DAR antagonist while the (-)-enantiomer was devoid of activity. Thus, the enantiomers of butaclamol are differentiated by the DAR with absolute enantiospecificity.

Another unique property of butaclamol is the restricted conformational mobility of its nucleus, a feature also possessed by (-)-apomorphine, II, a DAR agonist for which the DAR is equally enantiospecific (Saari, King and Lotti, 1973).

The molecular structures of these semi-rigid chiral ligands have been analyzed (Humber, Bruderlein and Voith, 1975) and a common chiral pharmacophore was identi-

fied, namely a phenyl ring and a nitrogen atom, uniquely oriented one to the other, and incorporated into an extended phenethylamine moiety. The same pharmacophore was also shown by Marshall and co-workers (1979) to be present in the DAR antagonist (+)-octoclothepin, III, as well as in other DAR ligands.

Humber, Bruderlein and Voith (1975) suggested that the phenyl ring and the nitrogen atom of the pharmacophore were responsible for butaclamol's activity by interacting with *primary binding sites* on the DAR.

Numerous butaclamol analogs have been investigated and results obtained with one of them, isobutaclamol, IV, will be described since it provides information on one aspect of DAR topography.

Isobutaclamol is the benzo[5,6]cyclohepta analog of butaclamol and its synthesis (Philipp, Humber and Voith, 1979), resolution (Humber and co-workers, 1979) have been described. The racemate and the enantiomers have been studied as DAR antagonists (Philipp, Humber and Voith, 1979) both *in vivo* (antagonism of amphetamine-induced stereotyped behaviour in rats) and *in vitro* (inhibition of ^{3}H-haloperidol binding by homogenized rat caudate nucleus), and the results are shown in Table I. It is apparent that (±)- and (+)-isobutaclamol are about equipotent as DAR antagonists, to (±)- and (+)-butaclamol both *in vivo* and *in vitro*.

Table I

DAR Antagonist Properties of Butaclamol and Analogs

Compound	Antagonism of ASB mg/kg, i.p. MED[a]	^{3}H-Haloperidol binding Inhibition IC_{50}, nM
(±)-butaclamol.HCl	0.62	3.5
(+)-butaclamol.HBr	0.31	1.4
(-)-butaclamol.HBr	> 50[b]	> 1000[c]
(±)-isobutaclamol.HCl	0.62	1.7
(+)-isobutaclamol.HBr	0.31	0.8
(-)-isobutaclamol.HBr	> 25[b]	> 1000[c]
(±)-anhydrobutaclamol.HCl	0.62	9.5
(±)-desoxybutaclamol.HCl	0.62	6.3

[a]Minimal effective dose antagonizing amphetamine stereotypy. [b]Highest dose tested. [c]Highest concentration tested.

Butaclamol's activity was ascribed to the presence of the neuroleptic pharmacophore within its extended phenethylamine moiety. Inspection of the structure of isobutaclamol reveals no such phenethylamine moiety, but instead, shows the presence of a phenylpropylamine moiety. It thus appears that butaclamol and isobutaclamol are incapable of presenting the same pharmacophore to the DAR. We have therefore studied the molecular structures of butaclamol and isobutaclamol more closely with a view to resolving this apparent anomaly.

The molecular structure and stereochemistry of (+)-isobutaclamol hydrobromide was determined by X-ray crystallography (Ahmed and Przybylska, 1979) and is shown in Fig. 1. It confirms all aspects of the constitution and assigned relative and absolute configurations at positions 3, 4a and 13a.

The conformation observed in the crystal structure, however, need not bear any relationship to that adopted by the molecule on interaction with the DAR; it is

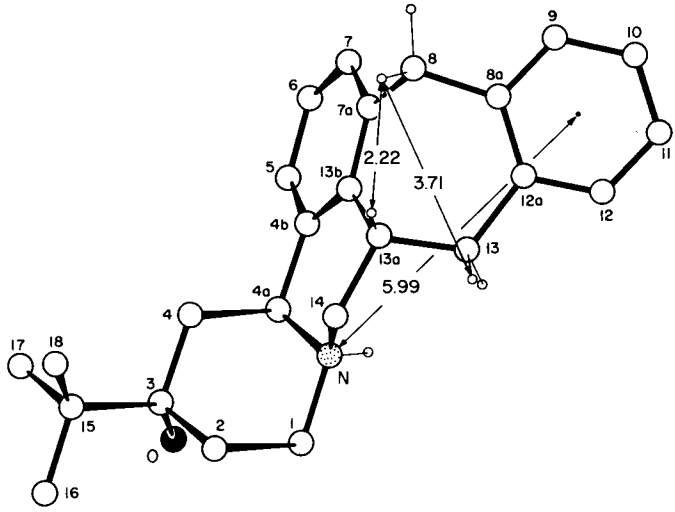


Figure 1. Crystal structure of (+)-isobutaclamol hydrobromide (the distances indicated in A are calculated from the crystallographic data)

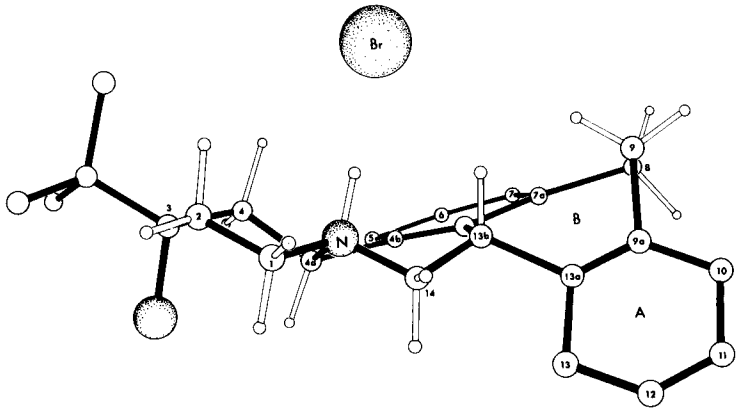


Figure 2. Crystal structure of (±)-butaclamol hydrobromide

necessary therefore to consider the nature of that conformation.

In the crystal, (+)-isobutaclamol hydrobromide has a *cisoid* fused rings DE quinolizine system, i.e., it has a 15S absolute configuration. In contrast, in the crystal structure of (±)-butaclamol hydrobromide (Bird, Bruderlein and Humber, 1976), Fig. 2, the quinolizine system has a *transoid* fusion. We have suggested previously (Philipp, Humber and Voith, 1979) that at the DAR, (+)-butaclamol and (+)-isobutaclamol exist predominately in the deprotonated forms. In these forms the nitrogen atom is achiral and the *cisoid* and *transoid* quinolizine species are readily interconvertible through nitrogen inversion. We suggest that on interaction with the DAR, (+)-isobutaclamol adopts a conformation with a *transoid* fusion of the quinolizine system. As will become evident in the sequel, only in that conformation can (+)-isobutaclamol present a pharmacophore which is capable of binding to the DAR.

In the crystal structure of butaclamol hydrobromide, Fig. 2, the cycloheptane ring exists in the conformation with eclipsed hydrogens at positions 9 and 13b (conformer A, Fig. 3). In order that (+)-butaclamol would be capable of presenting the same pharmacophore as does (-)-apomorphine, it was postulated that (+)-butaclamol, on interaction with the DAR, adopted the conformation with eclipsed hydrogens at positions 8 and 13b (conformer B, Fig. 3), rather than that observed in the crystal structure. In contrast, the conformation of the cycloheptane ring of (+)-isobutaclamol in the crystal structure has eclipsed hydrogens at positions 8 and 13a (conformer B, Fig. 4) rather than at positions 8 and 13 (conformer A, Fig. 4). We suggest that the cycloheptane ring of (+)-isobutaclamol retains its conformation B on interaction with the DAR.

When models of these deduced receptor-site conformations of (+)-isobutaclamol and (+)-butaclamol, in their conformations B are superimposed, Fig. 5, it is seen that the nitrogen atoms of the two ligands are coincident, but the phenyl rings A are not.

Thus, these agents, although closely related structurally, and interacting with a common receptor with affinities in the nanomolar range, do not possess a common pharmacophore.

Closer inspection of Fig. 5 reveals however that the rings A of the two ligands lie in the same plane and are immediately adjacent to each other, and suggests that there is a planar binding site on the DAR with the dimensions of at least two adjoining benzene rings, i.e., about 4.8 X 2.4 A. This binding site can accommodate the phenyl ring A of (+)-butaclamol and the catechol ring of (-)-apomorphine in the α-region, and can accommodate (+)-isobutaclamol's phenyl ring A in the β-region, while the nitrogen atoms of all three ligands are bound to the same site.

The precise relationship of the *aromatic binding site* and the *nitrogen location site* is illustrated, in Fig. 6, in terms of their XYZ coordinates. Measurements were obtained from models of conformers B of (+)-butaclamol and (+)-isobutaclamol, oriented in a cartesian coordinate system calibrated in Angstroms, such that the centers of rings A were located at points C and C', respectively, at a distance above the X-axis proportional to 4.7 A, and with the nitrogen atom located at point D in the -X+Z plane.

Other significant aspects also emerge from a consideration of this model of the DAR. We have suggested previously (Philipp, Humber and Voith, 1979) that (+)-butaclamol and (+)-isobutaclamol interact with the DAR in their deprotonated forms and that the nitrogen lone pair electrons participate in hydrogen bond formation with another electronegative atom.

Hydrogen bond formation is effective when the distance between the electronegative

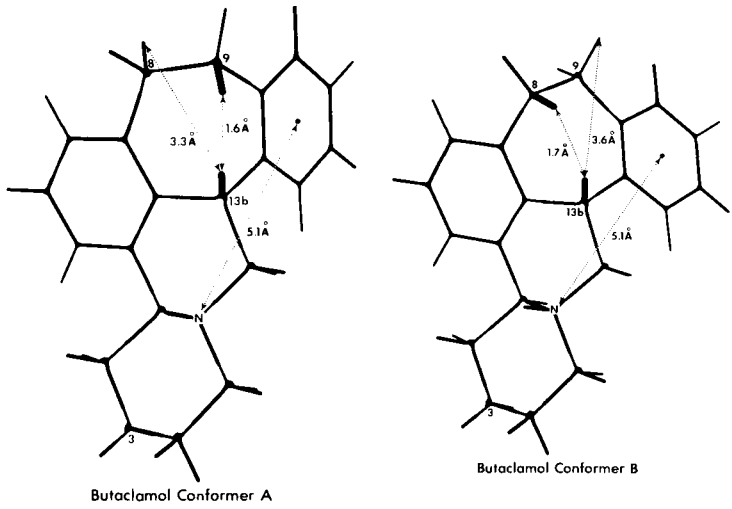


Figure 3. Shadowgraphs of Dreiding models of the nuclei of (+)-butaclamol conformers (the distances shown are from measurements on Dreiding models)

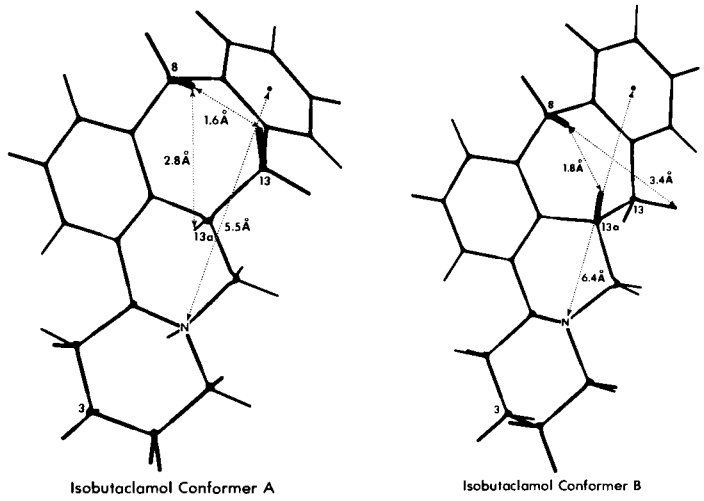


Figure 4. Shadowgraphs of Dreiding models of the nuclei of (+)-isobutaclamol conformers (the distances shown are from measurements of Dreiding models)

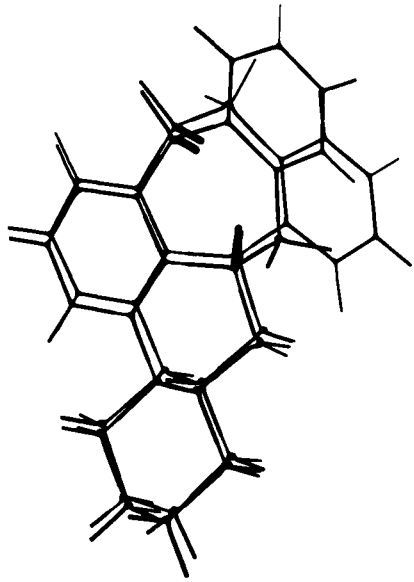


Figure 5. Superimposed shadowgraphs of Dreiding models of the nuclei of B conformers of (+)-butaclamol and (+)-isobutaclamol

atoms involved is between 2.5 and 2.7 A, and when the three atoms involved are linearly aligned. Consequently, we suggest that an electronegative atom is located at point G, 2.6 A from the nitrogen atom. Because of the rigidity of the butaclamol nucleus, the nitrogen lone pair electrons have a unique directional vector, which is fully defined by the value of 45° for the angle G D D' in Fig. 6. The point G has the coordinates -2.0 X and -1.8 Y and is located at the intersection of the -X-Y plane, and a perpendicular -X+Z plane which is 1.8 A (the distance O-F) below the plane in which the nitrogen atom is located. Thus the previously designated (Humber, Bruderlein and Voith, 1975) nitrogen atom *primary binding site*, must now be viewed as being composed of a *nitrogen location site* D and a complementary *hydrogen bond donor site* G.

This receptor representation also accounts for the chirality of the receptor. Thus, if the pharmacologically inactive (-)-enantiomer of butaclamol is oriented to the receptor with its ring A bound to the β-region of the *phenyl ring binding site*, it requires the existence of a *nitrogen atom location site* at position D' (+3.2 X, +0.9 Z). It would appear that either no such site exists at D', or, that the molecule would have to occupy "disallowed" space in the +Z octants of the coordinate system in which the receptor is oriented.

We have chosen, in Fig. 6, to orient the phenyl rings A of (+)-butaclamol and of (+)-isobutaclamol, both in conformation B, on the XY plane such that the nitrogen atoms are situated on the +Z side of the XY plane. Inspection of models of these ligands reveals the remarkable circumstance that with the exception of five hydrogen atoms of the former ligand (those attached to carbon atoms 1, 8, 9, 13b and 14), and the similar hydrogen atoms of the latter (attached to carbon atoms 1, 8,

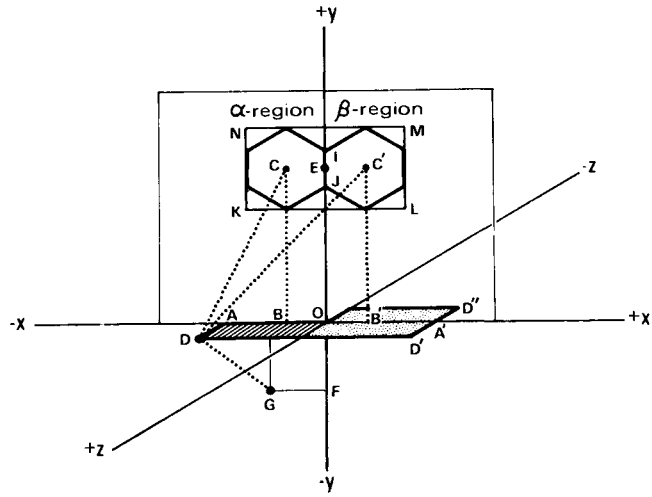


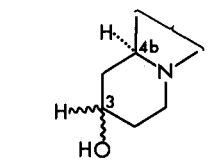
Figure 6. Representation of primary binding sites on the dopamine receptor derived from measurements on Dreiding models of the B conformers of (+)-butaclamol and (+)-isobutaclamol (see text). The figure is drawn to a scale of 1 cm = 1A. Key distances are: O-E = B-C = B'-C' = 4.7; A-D = A'D' = A''-D'' = 0.9; A-B = A'-B' = 2.0; A-B' = 4.4; D-C = 5.1; D-C' = 6.4; D-G = 2.6; A-O = A'-O' = 3.2; K-L = M-N = 4.8; N-K = M-L = 2.4; O-F = 1.8; F-G = 1.7.

13, 13a and 14), all the other fifty three atoms of the molecules lie either in the XY plane, or, on the +Z side of that plane. This observation suggests that the XY plane of the receptor represents an essentially flat membrane surface of which the phenyl ring *primary binding site* is a part.

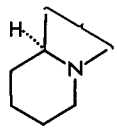
The ligands discussed above could, alternatively, have been oriented with their phenyl rings A bound to a complementary site on the XY plane, but with the nitrogen atoms located at position D'' in the -Z side of that plane. This alternative is identical to the first as long as no information is available concerning the nature of the proposed membrane that is represented by the XY plane. However, we consider it improbable that a receptor membrane would be so undifferentiated that specific ligand binding could occur, indiscriminately, on either face.

It was the presence of an extended phenethylamine moiety in (+)-butaclamol that prompted a comparison of its topography with that of the same grouping in (-)-apomorphine, and which led to the detection of common topographical features in the two ligands. (+)-Isobutaclamol does not contain such an extended phenethylamine moiety, but rather, a phenylpropylamine group constrained within a semi-rigid ring system. *A priori*, one might not have expected (+)-isobutaclamol to be capable of interacting with the receptor genetically designed for the phenethylamine neurotransmitter dopamine. However, because of the dimensions of the planar *phenyl ring primary binding site*, and because (+)-isobutaclamol and (+)-butaclamol have the same A-D distances they possess the essential features required for recognition by the DAR. This constitutes a unique instance where a phenylpropylamine grouping functions topographically as a phenethylamine moiety.

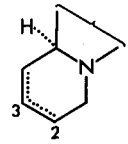
Amongst the numerous butaclamol analogs studied, a set of seven (V-XI), having modifications in ring E were prepared and evaluated as DAR antagonists (Humber and co-workers, 1979). Their structures are indicated below.



V 3H,4b-H- trans
VI 3H,4b-H- cis



VII



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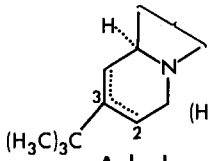
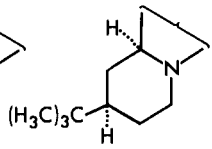
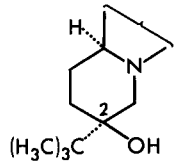


Anhydro-butaclamol
X



Desoxy-butaclamol
XI



VIII

Analog V which retains a 3-hydroxyl group but lacks the *tert*-butyl group, and its epimer VI, are inactive as DAR antagonists when evaluated *in vivo*. Similarly, analog VII, with both the hydroxyl and *tert*-butyl substituents absent, is inactive. Analog VIII has both the hydroxyl and *tert*-butyl substituents transposed to position 2 and is inactive, while analog IX, a mixture of unsubstituted Δ^2 and Δ^3 olefins, is also devoid of activity.

Of the series only two compounds, anhydrobutaclamol, X, a mixture of Δ^2 and Δ^3 olefins, and desoxybutaclamol, XI, retain potent DAR antagonist properties. These compounds were evaluated both *in vitro* and *in vivo* as described above, and the results are shown in Table I. It is apparent that they are qualitatively and quantitatively very similar to (±)-butaclamol and (±)-isobutaclamol.

Inspection of models of butaclamol and anhydrobutaclamol reveals that the rings E of the olefinic isomers can adopt only a single half-chair conformation. In both of these, the locus in space of the *tert*-butyl groups is very similar to the same group of butaclamol and the groups overlap to a considerable degree. Similarly, the locus of the volume occupied by the *tert*-butyl group of desoxybutaclamol, which was assigned an equatorial orientation on a ring E chair, is identical to that of the same moiety in butaclamol. In contrast, the centra of the volumes occupied by the *tert*-butyl groups of butaclamol and analog VIII, are separated by almost 3 A.

These findings suggest that DAR antagonist activity in compounds of the butaclamol series is critically dependent on the presence of a *tert*-butyl group attached equatorially at position-3 of the nucleus, and furthermore that a lipophilic *accessory binding site* must exist on the DAR macromolecule, which accommodates the *tert*-butyl groups of butaclamol, isobutaclamol, anhydrobutaclamol, and desoxybutaclamol.

The dimensions of the lipophilic *accessory binding site* cannot, at this stage, be precisely defined. From our previous studies it is known that an equatorially oriented group as small as ethyl (Bruderlein, Humber and Voith, 1975), or as large as a substituted phenyl ring (Voith, Bruderlein and Humber, 1978), will confer neuroleptic activity on the resultant analog. The *tert*-butyl group has dimensions close to those of a benzene ring.

The lipophilic *accessory binding site*, therefore, is viewed as a uniquely shaped cavity on a membrane surface having a minimum diameter of 2.5 A (Fig. 7).

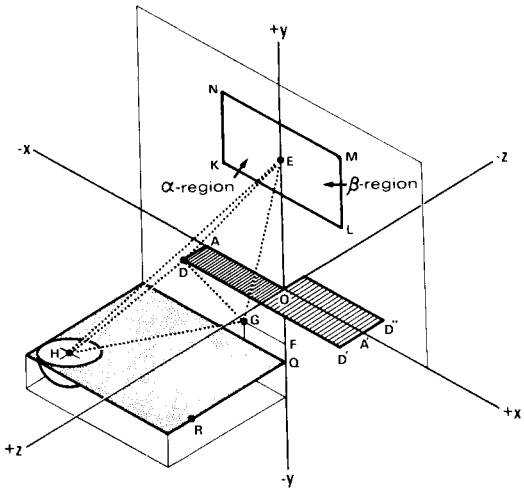


Figure 7. Representation of the lipophilic accessory binding site and the primary binding sites on the dopamine receptor (see text). The figure is drawn to a scale of 1 cm = 1 A. Key distances are: O-E = 4.7; A-D = 0.9; D-E = 5.7; A-O = 3.2; D-G = 2.6; H-E = 9.6; D-H = 4.5; O-F = 1.8; O-Q = 2.5; H-G = 6.5; Q-R = 5.0; H-R = 4.0; F-Q = 0.7; N-K = M-L = 2.4; K-L = M-N = 4.8.

Measurements on a model oriented in a cartesian coordinate system calibrated in Angstrom units shows that it is centered at the point H (+4.0 Z, -5.0 X, -2.5 Y) in a -X+Z plane parallel to that in which the nitrogen atom is located. The receptor representation in Fig. 7 shows that it is 4.5 A (D-H) from the *nitrogen location site* D, 6.5 A (H-G) from the *hydrogen bond donor site* G, and 9.6 A (H-E) from the center of the *phenyl ring binding site* E.

While active compounds of the butaclamol series must utilize this *accessory binding site*, it appears that it is not utilized by any other known DAR antagonist or agonists. It is probable that other *accessory binding sites* exist on the DAR macromolecule. Their identification would require a detailed study of the essential structural and stereochemical requirements for activity in other series of DAR agonists and antagonists.

These key distances and coordinates involving the lipophilic *accessory binding site* H, along with the distances and coordinates related to the *nitrogen location site*

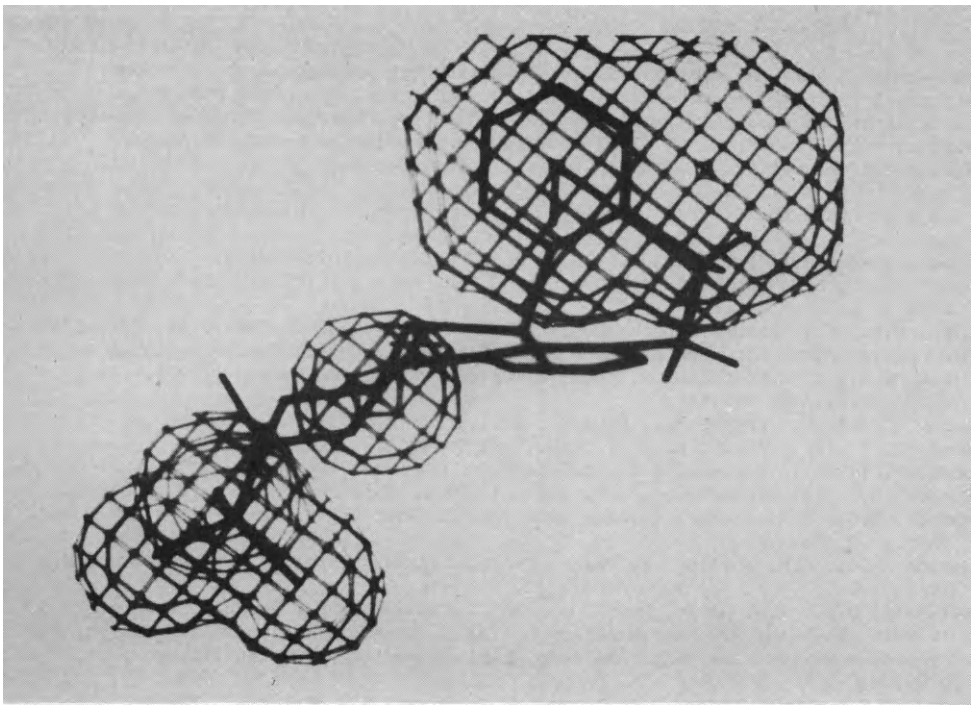


Figure 8. Model of the dopamine receptor, with the ( )-butaclamol molecule superimposed, generated by the MMS-X computer based molecular modeling and graphic display system. (Courtesy of Dr. G. Marshall, Washington University, St. Louis).

D, the *hydrogen bond donor site* G, and the α and β regions of the *phenyl ring binding site* represent a comprehensive preliminary description of the topography of the DAR.

Fig. 8 is another representation of the receptor model, generated with the MMS-X computer-based molecular modeling, and graphic display system (Barry and co-workers, 1974). Input data comprised the XYZ coordinates of those atoms of (+)-butaclamol and (+)-isobutaclamol, in their receptor-site conformations, which served to define the α- and β-regions of the *aromatic binding site*, the *nitrogen atom location site*, and, the *lipophilic accessory binding site*. The coordinates were obtained either from crystallographic results, or, from a program which generates coordinate data from bond lengths and angles. The display, Fig. 8, is in the form of an electron density map of the atoms referred to above, contoured to their van der Waals radii (light lines). The display also shows the molecule of (+)-butaclamol (heavy lines) superimposed on the receptor model.

In conclusion, from the study on the relationship between DAR antagonist activity and detailed molecular structure in certain analogs of butaclamol, it has been possible to confirm the existence of a lipophilic *accessory binding site* on the DAR, to define its probable dimensions, and to specify its locus with respect to the phenyl ring and the nitrogen atom *primary binding sites*.

ACKNOWLEDGMENTS

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Multiple Analgesic Receptors

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ABSTRACT

Neurophysiological observations support the hypothesis that morphine and benzomorphan analogs interact with several types of receptors (μ to morphine, K to Ketocyclazocine and σ to N-allyl-nor-phenazocine) leading to different pharmacological profiles. In addition on the basis of the results obtained in four assay models it appears that opioid peptides act on two types of receptors (μ and δ). It is of interest to note that the opiate peptide receptors do not fit into the classification for opiate alkaloid receptors suggesting that opiate peptides may subserve in addition to analgesia other physiological functions. In this review, the effects of opioid peptides in stress, analgesia, blood pressure regulation and epilepsy are analysed.
On the other hand it has been shown that the alpha adrenoceptor agonist clonidine elicites analgesia by a mechanism which is not sensitive to naloxone. Opiates and alpha-2 adrenoceptor agonists selectively inhibit neuronal firing as well as neurotransmitter release from catecholaminergic and cholinergic nerve terminals in the cortex. The in vitro results suggest that one neuron may possess several types of receptors mediating analgesic effects. These observations are compatible with the hypothesis of "mobile" receptors, according to which a number of receptor specific agonists can independently stimulate a unique cellular efector.

KEY WORDS

Opiate receptors, Analgesic receptors, Clonidine, Calcium, Cyclic nucleotides, stress, blood pressure, epilepsy.

I. New insights on the drug-receptor interaction.

The chemical theory of synaptic transmission implies the existence of a specific chemical mechanism, which greatly amplifies the electrical signal travelling down the nerve terminals and which results in the response of the peripheral or central postsynaptic component. The existence of a postsynaptic receptive substance with affinity for drugs was first postulated by Langley in 1909 . In addition to the postsynaptic receptors that mediate the response of the effector organ or the postsynaptic neuron response, the presence of presynaptic receptors localized in nerve terminals that regulate the calcium dependent neurotransmitter release has been reported (Langer, 1978).
The use of the term "receptor" is an attempt to explain the actions of drugs on tissues, but it also reflects our ignorance of the molecular characteristics of the drug locus of action. In a few cases, when this locus is known, we no longer refer to it as the receptor, but rather by its chemical nature (Cuatrecasas, 1975).
According to Goldstein (1974), a pharmacological effect depends upon the affinity and intrinsic activity of the drug for the receptor, the allosteric state of the receptor and the number of spare receptors contained in the tissue. One such receptor possess two distinct functions: 1) affinity or ability to recognize the specific ligand and 2) intrinsic activity or capacity to translate the interaction with drugs into pharmacodynamical effects.
It was not until 1970 that the interaction of a hormone with its receptor was studied directly. In that year, two independent groups using the radioactively labeled hormones ^{125}I-adrenocorticotrophic hormone (ACTH) and ^{125}I-angiotensin respectively, demonstrated the existence of specific membrane binding sites (Lefkowitz et al. 1970 and Lin et al. 1970). In the next few years, the approach was extended to the study of a variety of peptide hormones and nicotinic cholinergic receptors (Kahn, 1974 and Karlin, 1974).
Terenius (1973), Simon et al. (1973) and Pert and Snyder (1973) using high specific radioactive opiates demonstrated unequivocally the existence of stereospecific binding sites for alkaloid derived opioids in the brain. This finding was followed by the isolation from the brain of 3 natural peptides with opiate properties named enkephalins and endorphins (Hughes, et al. 1975).
The binding of radioligands to membrane sites is surmised to reflect the interaction with specific receptors provided that the following requirements are met: a) strict structural and steric specificity, b) saturability, which indicates a limited number of binding sites, c) tissue specificity in accordance with biological target cell sensitivity, d) high affinity in harmony with the physiological concentrations of the neurotransmitter, and e) reversibility which is kinetically consistent with the reversal of the physiological effects upon removal of the hormone or neurotransmitter from the medium.
Drug receptor binding studies can be deceptive because the possibility exists that the binding sites are artefactual and therefore do

not represent true receptors, but merely saturable binding to non-specific sites on filters or tissues. Thus, certain filters and lipids can bind opiates in a stereospecific fashion with optical isomers displaying the same relative potency of that found at the opiate receptor (Snyder et al. 1975); a similar test has been observed with some catecholamines (Williams et al. 1978) and with Phencyclidine(Snyder, 1980).
Burgen (1979) put in doubt the existence of a unique binding site for both agonists and antagonists and suggested the existence of two different conformations for the same receptor: one, the ground state which binds the antagonist and the other, the excitated state which binds the agonist. This concept is in agreement with the observation that in the presence of sodium salts in the incubation medium, the binding of agonists is inhibited, whereas the binding of antagonists is increased (Pert and Snyder, 1973). A possible explanation to this finding is that the rate of dissociation from the opiate receptor is markedly increased in the presence of sodium chloride at least for the agonist etorphine (Simon et al. 1975). An additional differentiation between the binding of agonists and antagonists has been demonstrated using guanine nucleotides. In the presence of sodium only negligible effects of GTP on the binding of the antagonists ^{3}H-naloxone and ^{3}H-diprenorphine are shown, but under similar experimental conditions a marked reduction in the binding of ^{3}H-opiate agonists has been observed (Snyder et al. 1978). The concept of a mobile or floating receptor model proposed by Cuatrecasas (1974) brought a new idea about receptors. According to this receptor-model, the membrane seems to contain two kinds of components: A) stereospecific recognition sites (aceptors), which are macromolecules with affinity and specific availability to bind the ligands, B) effector molecules such as adenylciclase, guanylciclase, enzymes, ionophore-proteins, etc. which trigger the chain of events that lead to the pharmacological effects by causing an immediate change of membrane function. The dual function of the recognition plus the effector action really constitutes the true receptor. The ligand-receptor interaction implies two different kinds of kinetics: 1) the formation of complexes between the neurotransmitter and the aceptor site and 2) the formation of a full active ternary complex between the ligand-aceptor complex and the different effector macromolecules of the cell membrane (Hollenberg, 1978). Each ligand-aceptor complex may exhibit different affinities toward the various membrane effectors. Thus, a unique membrane effector responsible for analgesia effects may be activated by different ligand-aceptor complexes with similar affinities. However, the affinities of these complexes for the effectors mediating functions such as respiratory rate, cardiovascular tonus, etc. may be different.
Finally De Robertis (1975) underlined the nature of some possible events following the conformational cnanges in the neuronal membrane: Ca^{2+} displacements, metabolic changes mainly affecting cAMP, cGMP and phosphatidyl-inositol, changes on the conductance of the membrane and ionic translocations on the membrane. It is of interest to note the important role played by Ca^{2+} in a variety of cellular enzyme systems. This role is rather similar to the regulatory actions of cyclic nucleotides. The functional role of Ca^{2+} appears to be mediated by Calmodulin an ubiquitous calcium-binding protein that can activate a variety of enzymes including adenylate cyclase, cyclic nucleotide, phosphodiesterase and proteinkinases. Calmodulin may be acting as a multiple acceptor protein mediating the specific actions of calcium (Dedman et al. 1977, Means and Dedman, 1980).

II. Opiate-Receptors.

Physiological experiments carried out on the chronic spinal dog provide evidence in favour of 3 classes of opioid receptors a "μ -receptor" to mor-

phine which is responsible for a supraspinal type of analgesia, feelings of well-being and at least an important part of morphine-type physical-dependence; a " κ-receptor" to Ketocyclazocine which mediates spinal analgesia, sedation, anesthesia and other signs of cyclazocine-dependence; and a " σ-receptor" to N-allyl-norphenazocine (SKF 10.047) which is responsible for feelings of disphoria, mydriasis and respiratory stimulation (Martin et al. 1976).
Lord et al. (1977) and Kosterlitz (1978) established 4 models of assays: the mouse vas deferens, and guinea-pig ileum preparations, and the ^{3}H-leu-enkephalin, and ^{3}H-naltrexone binding tests on guinea-pig brain membranes.

The ratio between the inhibitory effect of opiates on the electrically-induced contractile responses of the guinea-pig ileum (mainly μ-receptors) and on the mouse vas deferens (mainly δ-receptors) was: 0.11 for Met-enkephalin, 1.2 for beta-endorphin and 6.3 for morphine. Similarly, morphine displays a very low affinity at the ^{3}H-leucine-enkephalin binding site whereas its affinity at the ^{3}H-naltrexone binding site is of the same order of magnitude as that of the enkephalins.

On the basis of these assays the pressence of a new type of opiate receptor in the mouse vas deferens has been postulated. The δ-receptor is pharmacologically different from the μ-receptor which is much more sensitive to the Met- and Leu-enkephalin than to morphine.

In the mouse vas deferens, δ-receptors may be closely related to the 3H-leucine-enkephalin binding sites in the guinea-pig brain.

In central nervous system (CNS) morphine effects appear to be mainly mediated by two classes of receptors:

1) a naloxone-sensitive endorphine-receptor (μ-receptor) which shows stereospecific affinity for opioids and mediates the analgesic and catatonic effects of morphine;

2) a second receptor, naloxone-insensitive which does not show stereospecific affinity for opioids and mediates the hyperexcitability (explosive motor behaviour, EMB) induced by morphine.

It has been suggested that opiate abstinence syndrome might be due to morphine exciting the EMB-receptor after naloxone blockade of the endorphin receptor (Jacquet, 1978). Jacquet recently reported that the injection of ACTH in the periaqueductal grey matter (PAG) of opiate-naive-rats results in a behavior similar to that observed in the opiate-abstinence syndrome. This finding suggests that ACTH could be the endogenous ligand of the EMB-receptor. In conclusion, at least in the PAG, two kinds of effects mediated by opiate receptors have been described: 1) analgesia induced by beta-endorphin as an endogenous ligand, 2) EMB induced by ACTH as an endogenous ligand.
It is worth noting that the term opiate-receptors does not exclusively refer to analgesic receptors. In the classification from Martin et al. (1976), the sigma-receptors specific to SKF-10.047 do not produce analgesic effects. In the same way, the EMB-receptors (explosive motor behavior) from Jacquet (1978) are morphinic receptors, but not analgesic.
The receptor-dualism concept was based on the observation that low doses of nalorphine antagonized the analgesic action of morphine, whereas higher doses increased the analgesic activity of morphine (Houde and Wallestein, 1956). As a result of the combination of nalorphin with fixed doses of morphine, a biphasic dose-response curve was obtained. Martin (1967) coined

the concept of nalorphine receptor-dualism so as to explain the morphine-nalorphin interaction in terms of several independent categories of analgesic receptors.

Accordingly, the term agonist-antagonists as applied to the actions of opiate-analgesics might have two possible meanings: 1) the concept of multiple receptors and receptor-dualism, and 2) the concept of partial agonism. Thus, an opiate agent may act as a selective agonist (either partial or strong) on a particular type of opiate receptor such as μ , K, σ and δ . In addition to the agonist properties, an opiate agent can behave as an antagonist (either partial agonist or competitive antagonist) at the same or at another type of opiate-receptor.

In order to classify the opiate agents, one must bear in mind that most pharmacological agents are dirty drugs, so any classification we can do may only consider preferential actions of drugs. Accordingly, the opiate drugs have been classified in the following groups: I. Pure agonists II. Partial agonists of morphine, III. Partial agonists of nalorphine, IV. Pure antagonists (Martin, 1979; Houde, 1979 and Creese et al. 1979). Tables I and II show some relevant pharmacological characteristics of each group.

TABLE I. CLASSES OF OPIATE RECEPTORS

Martin et al, 1976; Kosterlitz, 1978; Martin, 1979; Jacquet, 1978; Frenk et al. 1978 and Cowan et al. 1979.

RECEPTOR	EFFECT	AGONIST	BINDING-SITE LOCALIZATION	ANTAGONIST
MU μ	ANALGESIA ANTIEPILEPTIC HYPOTENSION BRADYCARDIA ↓ RESPIRATION ↓ RATE MIOSIS HYPOTHERMIA	MORPHINE ETORPHINE PHENAZOCINE β ENDORPHIN	DOG SP.-CORD GUINEA-PIG IL. RAT BRAIN PAG	BLOCKED BY LOW DOSES OF NALONE AND NALTREXONE
KAPPA K	ANALGESIA CONVULSANT MIOSIS BRADYCARDIA NO ↓ RESP.RATE NO ↓ TEMPERATURE	KETOCYCLAZOCINE NALORPHINE	DOG SP.-CORD	NOT AFFECTED BY NALOXONE
DELTA δ	SEDATION POOR ANALGESIA CONVULSANT TACHYCARDIA VASOCONSTR. HYPERTENSION	LEU-ENK MET-ENK D-ALA^2-D. LEU^5-ENK	D. MEDIAL N. THALAMUS M.V. DEFERENS RAT BRAIN	BLOCKED BY HIGH DOSES OF NALOXONE
SIGMA O	NO ANALGESIA DELIRIUM ANTIEPILEPTIC MIDRIASIS TACHYCARDIA RESP.FREQ.	SKF 10047 N-ALLYL-NOR-FENAZOCIN CYCLAZOCIN	DOG SP. CORD	NOT AFFECTED BY NALOXONE
EMB	EXPLOSIVE MOTOR BEHAVIOR	MORPHINE AFTER NALOXONE	PAG	FACILITED BY NALOXONE
?	CONVULSANT	MEPERIDINE PENTAZOCINE	RAT BRAIN	FACILITED BY NALOXONE

TABLE II. CLASSIFICATION OF OPIATE AGONISTS

CHEMICAL GROUP	RECEPTOR TYPE	SELECTIVE AGONISTS
MORPHINE ANALOGUES	"μ"	MORPHINE OXYMORPHONE FENTANYL Na^+ ratio>30 NORMORPHONE DIHYDROMORPHINE
		ETORPHINE LEVORPHANOL Na^+ ratio<30 PHENAZOCINE
	"K"	KETOCYCLAZOCINE
	"σ"	N-ALYL-NOR-PHENAZOCINE (SKF-10047)
OPIATE PEPTIDES	"δ"	LEU-ENKEPHALIN MET-ENKEPHALIN D.ALA^2-D.LEU^5-ENK. (Wellcome)
	"μ"	β-ENDORPHINE D.ALA^2-ME.PHE^4-MET(O)-OL^5-ENK. (Sandoz)

The actions and interactions among narcotic agonists and antagonists are complex and not fully understood; nevertheless, the new concepts on different types of opiate-receptors provide a basis for understanding some apparently paradoxical effects (Houde, 1979). In addition, opioid agonists as well as narcotic antagonists exert their analgesic effects as a result of the receptor occupation with different degrees of affinity and intrinsic activity: 1) Morphine and surrogates are assumed to act almost solely on the "μ-receptors" and to possess poor affinity for and intrinsic activity at the "K and σ-receptors"; 2) Pure-antagonists such as naloxone and naltrexone appear to possess different degrees of affinity to each one of the opiate receptors, but without intrinsic activity for all of them; 3) Mixed agonists-antagonists or partial agonist of the morphine type exhibit pharmacological properties similar to those of morphine and act on "μ-receptors" in order to produced their analgesic effects; 4) Mixed agonists-antagonists or partial-agonists of nalorphine are considered to have varying affinities and intrinsic activity at all 4 receptors, but are believed to exert their analgesic actions primarily by occupation of "K-receptors".

III. Mechanisms of analgesia.

A.- The role of calcium as a second messenger in the action of opiates has become a current subject of research. It has been reported that morphine can not inhibit respiration in brain slices unless the Ca^{2+} concentration is greatly reduced (Takemori, 1962; Elliott et al. 1963). Following a single dose of morphine, a reduction in the levels of Ca^{2+} in intact synaptosomes has been shown (Cardenas and Ross, 1976). This effect could be due to morphine inhibition of the synaptosomal $^{45}Ca^{2+}$ uptake (Guerrero-Muñoz et al. 1979). The inhibition of $^{45}Ca^{2+}$ uptake by morphine is a specific effect as indicated by the fact that it can be prevented by naloxone. Moreover, in contrast to levorphanol, $^{45}Ca^{2+}$ uptake is not affected by the inactive stereoisomer dextrophan (Guerrero-Muñoz et al. 1979). In lysed synaptosomes, morphine was shown to decrease $^{45}Ca^{2+}$ uptake only in the presence of ATP; thus suggesting that the drug affects an active process (Guerrero-Muñoz et al. 1979).

The in vitro results are in agreement with the fact the antinociceptive response of morphine was antagonized after intracerebroventricular (ICV) injection of Ca^{2+} in mice; whereas, it was potentiated by the Ca^{2+} chelator EGTA (Harris et al. 1975). On the other hand, Lanthanum ions (La^{3+}) have been found to inhibit the binding and transport of Ca^{2+} in a variety of tissues (Weiss, 1974). The ICV administration of La^{3+} in mice potentiates morphine analgesia; whereas La^{3+} alone elicits dose dependent antinociceptive responses. The effect of La^{3+} can be antagonized by both ICV Ca^{2+} and naloxone (Iwamoto et al. 1978).
In order to explain the interaction between morphine and brain calcium, it has been suggested that Ca^{2+} and opiate-receptor binding sites are closely located and may be functionally linked through subunit interactions (Ross, 1977) as shown in Fig. 1. However, the possibility exists that Ca^{2+} interference with morphine inhibition might take place separatly from the opiate receptor.

B.- Several lines of evidence suggest that the cyclic nucleotide system mediates some opiate effects in the brain subsequent to the specific binding of opiate ligands to the neuronal receptors (Daly, 1976; Nathanson, 1977; Greengard, 1978). It has been shown that therapeutic concentrations of morphine inhibit PGE_1 or PGE_2-stimulated adenylate cyclase activity in homogenates of rat brain. This effect is completely blocked in the presence of naloxone (Collier and Roy, 1974). Similar results have been obtained in neuroblastome x glioma hybrid cell preparations (Klee and Niremberg, 1974). However, in contrast to most tissues, morphine increases adenylate cyclase activity in homogenates of caudate nucleus (Puri et al. 1975). Consistent with the results of Collier and Roy (1974), the intracerebral injection of cyclic adenosine 3'5'-monophosphate (cAMP) in mice antagonizes morphine analgesia. This effect was mimicked by the dibutiryl derivative of cAMP and theophylline (Ho et al. 1973). In addition, systemic administration of phosphodiesterase inhibitors or intraventricular injections of cyclic AMP intesifies the abstinence syndrome induced by naloxone (Collier and Francis, 1975; Mehta and Johnson 1974). Only recently have investi gations been aimed at assessing the relationship between opiate effects and cyclic guanosine 3'5'-monophosphate (cGMP) and up to now, the results are somewhat controversial.
Racagni et al. (1976) and Minneman and Iversen (1976) respectively reported evidence that narcotic analgesics increase cGMP accumulation in rat striatum under in vivo and in vitro experimental conditions. In contrast, it has been shown that the acute administration of morphine produces a dose-dependent reduction in cGMP levels in the PAG of the rat (O'Callaghan et al. 1979). Whether direct or indirect mechanisms are involved in the actions of morphine on cGMP levels has not been established. Since the synthesis of cGMP in the brain appears to be regulated by intracellular calcium ions (Ferrendelli et al. 1976), the influence of opiates on cGMP levels might be explained by an ionic mechanism. The intracerebroventricular administration of the dibutyryl derivative of cGMP in the rat produces a dose-related analgesia which is not sensitive to naloxone. The evidence obtained from the injection of dibutyryl cGMP in different brain areas suggest that endogenous cGMP does not mimick nor share a common mechanism of action with morphine (Cohn et al. 1978).
C.- The studies in cardiac muscle indicate that during membrane excitation a slow current of calcium (iCa^{2+} or iSi) is produced. This current iSi was modified by beta-adrenergic and muscarin-cholinergic transmitter and related drugs; while beta-agonists increases "iSi", muscarin agonists decreases it, (Ten Eick et al. 1976 ; Reuter 1979). These effects can be mimicked by cAMP (adrenergic effects) or by cGMP (cholinergic effects) (Tsien 1977). Harald Reuter in 1979 suggested that in the voltage sensitive chanels, iSi is regulated by the ratio cAMP/cGMP in the cardiac cells. In our laboratory Ramon et al. (1980) using the voltage-clamp technique in sheep isolated Purkinje fibers, found that morphine (10^{-6}M) decreased the action potential

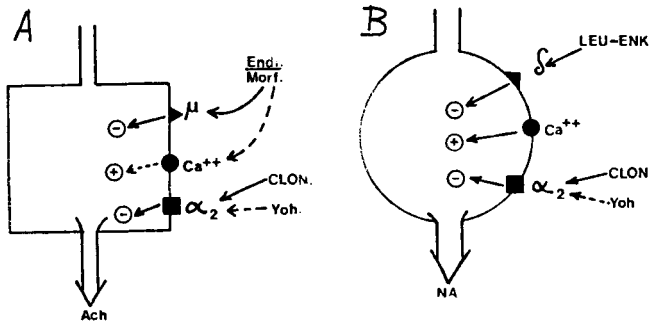

Fig. 1. Presynaptic modulation of neurotransmitter release in A) the guinea-pig ileum (Ach) and in B) mouse-vas deferens (NA).

duration, abolished the spontaneous activity and membrane oscillations of the plateau phase and caused a very clear reduction of iSi. We suggest that the arrival of an impulse to the nerve terminal produces a wave of depolarization causing an influx of Ca^{++}. This influx of Ca^{2+} in the nerve terminals preceding the neuro-transmitter release could be equivalent in biophysical terms to the iSi observed in cardiac fibers. In the CNS a reduction of Ca^{2+} by morphine similar to the iSi effects in the heart could explain the morphine inhibition of the synaptosomal $^{45}Ca^{2+}$ uptake.

D.- A number of drugs other than opiates have been reported to produce analgesic effects on the CNS. The analgesic properties of cholinesterase inhibitors and parasympathomimetic agents are well known (Takemori et al. 1975; Pedigo et al. 1975). Recently it has been shown that the alpha$_2$-adrenoceptor agonist, clonidine, is 5-60 times more potent than morphine in various analgesic assays (Fielding et al. 1978). Clonidine increased the latency of the tail-flick response in a dose-dependent manner and this effect was not prevented by naloxone (Spaulding et al. 1979).
A role of biogenic amines in narcotic analgesia has been postulated on the basis of the antagonism of morphine analgesia by reserpine (Schauman, 1957; Takagi et al. 1964; Rudzik and Mennear , 1965). Moreover, cholinolytic drugs such as atropine and hemicholinium have been reported to inhibit morphine analgesia (Takemori et al, 1975; Bhargava et al. 1974).
Consequently the actions of analgesic agents like opiates and clonidine on 1) catecholaminergic and 2) cholinergic neuronal system has been reviewed.

1) In the rat central noradrenergic nerve terminals, presynaptic alpha-autoreceptors mediate a negative feed-back mechanism by which noradrenaline or clonidine inhibit the release of the neurotransmitter (Starke, 1977). In addition, beta-endorphin and morphine reduce the stimulation-evoked release of ^{3}H-noradrenaline from the rat cerebral cortex while these effects were prevented by naloxone (Taube et al, 1976; Arbilla and Langer, 1978). Moreover, the degeneration of noradrenergic neurons within the locus coeruleus is accompanied by a significant reduction in the number of ^{3}H-naloxone binding sites in cerebral cortex and cerebellum (Schwartz et al. 1978). These results are compatible with the presence of inhibitory presynaptic opiate receptors as well as presynaptic alpha-autoreceptors on central noradrenaline nerve

terminals. The locus coeruleus represents the largest cluster of norepinephrine containing neurons in the central nervous system (Dahlstrom and Fuxe, 1965).In locus coeruleus neurons, there is an inhibitory met-enkephalin input which acts upon dendrite and/or cell body opiate receptors as well as the alpha$_2$-adrenergic autoreceptors which appear to mediate the collateral inhibitory responses (Young et al. 1977). In morphine-dependent rats, naloxone has been shown to induce a marked activation on the rate of firing of the locus coeruleus cells. Under experimental conditions in which opiate receptors are blocked, clonidine may suppress the naloxone-induced cell firing. This finding is compatible with clonidine activating a non-opiate inhibitory receptor and supports the view that opiates and clonidine interact with different inhibitory receptors on the locus coeruleus neurons (Aghajanian, 1978; Aghajanian and Cedarbaum, 1979). In addition, these results suggest that the opiate withdrawal syndrome may be due, in part, to increased activity in the noradrenergic neurons of the locus coeruleus, which are regulated by both alpha$_2$-adrenoceptors and opiate receptors.
It has been reported that exposure to morphine metionine-enkephalins and beta-endorphin reduced the depolarization-evoked release of ^{3}H-dopamine from striatal slices in a naloxone-sensitive manner (Loh et al. 1976; Subramanian et al. 1977). In contrast, Arbilla and Langer (1978) failed to confirm the inhibitory effect of morphine and beta-endorphin in release studies. The microinjection of 6-hydroxydopamine into the substantia nigra produces an almost total degeneration of dopaminergic nerve terminals in the striatum. Under these experimental conditions,, there is a significant decrease in the number of 3H-leu-enkephalin and 3H-naloxone binding sites in the striatum as well as in the substantia nigra. The binding results suggest the existence of opiate receptors localized in the dopaminergic nerve terminals of the striatum and in the dopaminergic cell bodies or dendrites of the substantia nigra. In contrast, it should be mentioned that clonidine has no direct effect on dopamine turnover (Anden and others , 1976) and this finding is consistent with the observations of Aghajanian and Bunney (1977), who showed that clonidine had no depressant effect on dopaminergic neurons.

2) It is well known that morphine inhibits the stimulation-evoked release of acetylcholine in the longitudinal muscle myenteric plexus of the guinea-pig ileum (Cox and Weinstock,1966); while naloxone facilitates this release (Waterfield and Kosterlitz, 1975). These results support the existence of inhibitory presynaptic opiate receptors on peripheral cholinergic neurons. Moreover, exposure to the alpha-adrenergic agonists, noradrenaline and clonidine, also inhibits the release of acetylcholine from the myenteric neurons by activating presynaptic alpha-adrenoceptors (Paton and Vizi, 1969; Scriabine et al, 1970; Wikberg, 1978). A similar inhibitory effect of noradrenaline and clonidine has been observed in the ouabain-evoked acetylcholine released from cerebral cortex slices (Paton and Vizi, 1969), but not in the acetylcholine released from striatal slices (Vizi et al. 1977). On the other hand the effect of opiates on the release of acetylcholine at central cholinergic synapses is somewhat controversial. While some authors such as Beleslin and Polak (1965) have observed an inhibition of acetylcholine release after morphine, others have found both inhibitory and facilitatory effects depending upon the dose of the opiate and the animal species used (Domino, 1975). The above results provide evidence for the existence of opiate-sensitive neurons utilizing noradrenaline, dopamine and acetylcholine as neurotransmitters. Inhibitory opiate as well as alpha$_2$-adrenergic receptors may be localized presynaptically on noradrenergic and cholinergic nerve terminals, but not in the dopaminergic ones. On the other hand, both inhibitory receptors have been demonstrated in the soma-dendrite regions of noradrenergic neurons. It is worth noting that clonidine and opiate agonists, acting at specific separate receptors, may inhibit the rate of firing and/or the neurotransmitter released from the

same neuron. The relevance of these observations would be that drug activation of other receptors (such as alpha$_2$-adrenergic receptors) may interfere with central neurotransmission in a similar way like opiate drugs. Thus, the possibility of developing new analgesic drugs lacking the disadvantages of opiate drugs exists.

IV. Clinical and therapeutic relevance of opiate receptor research.

The main disadvantage observed during the clinical use of analgesic opiates is their ability to induce tolerance and dependence. This is particularly true of pure opiate agonists which can be biochemically characterized by a decreased affinity (15-100 fold) at the opiate receptor binding sites in the presence of high concentrations of sodium. The mixed agonist-antagonists were considered as a different class of opiate drugs, which do not seem to cause addiction as readily as pure opiate agonists. Most of these drugs belong to the class of compounds known as the benzomorphans and offered a promise as relatively non-addictive pain relievers. However, although drugs like pentazocine are less addictive than conventional opiate agonists, they do not relieve severe pain as well as morphine. In addition, at therapeutic doses, pentazocine sometimes elicits anxiety, depersonalization and psychotomimetic effects. Recently a new group of mixed agonist-antagonists of opiates, which in contrast to pentazocine act at the same receptor as morphine, has been developed.Buprenorphine is the most representative drug of this group of partial agonists of morphine and is considered to be 25-40 fold as potent as morphine as an analgesic. The main advantage that buprenorphine exhibits is the absence of physical dependence after its use and because of that, it should be a new and effective drug for heroin addicts, (Mello et al. 1980). The isolation and identification of opiate peptides was assumed to constitute an important development in order to obtain therapeutically useful analgesics. Several modifications in the molecular structure of enkephalins has made it possible to obtain derivatives which are orally active analgesics (Roemer et al. 1977). One of this compounds, FK-33824, also elicits physical dependence in some animal tests, but is less addictive relative to the conventional opiate agonists. Another source of potential therapeutic analgesics is the enkephalinase inhibitors. Enkephalinase is considered to be a specific peptidase whose brain regional distribution is similar to that of the opiate receptors (Malfroy et al. 1978). Enkephalinase seems to be pharmacologically similar to the angiotensin converting enzyme (Swerts et al. 1979). Recently, it has been shown that SQ 14225, an inhibitor of the angiotensin converting enzyme, potentiates the analgesic effect of morphine (Turker et al. 1979).
In spite of the advances obtained in developing new opiate drugs lacking addiction potential, a new line of research in the field of analgesia should consider non-opiate receptors as potential targets for new analgesic drugs. It has been shown that drugs which block the alpha$_2$-adrenergic receptors, such as piperoxan and yohimbine, accelerate the firing of locus coeruleus neurons and produce symptoms which resemble opiate withdrawal. Unquestionably, there may be some pharmacodynamical-relation between clonidine and morphine and the latter's analogues. As anteriously was mentioned clonidine analgesic activity was reversed by yohimbine, but not by naloxone in doses of 10 mg/kg while the analgesic effect of clonidine by morphine was increased. No cross-tolerance was observed between morphine and clonidine. These data indicate that clonidine induced analgesia is not a result of an interaction at morphine receptors; but rather common pathways are present which appear to complement the agonist interactions of each one. Clonidine was effective in humans by reversing the withdrawal syndrome after discontinuance of methadone administration in opiate dependent addicts (Gold et al : 1978) and this therapeutical effect has been confirmed experimentally in mor-

phine-dependent animals (Gilan et al. 1979; Crawley et al. 1979). The withdrawal syndrome in rats produced by Fentanyl was blocked by clonidine, too.
Some aspects of opiate research with clinical relevance will be dealt with briefly in the following sections: 1) Opiates and $alpha_2$ adrenergic receptors. 2) Stress and endogenous opiates. 3) Peptidergic system and blood pressure. 4) Opiate receptors and epilepsia.

1) Fuxe, Hokfelt et al. (1975) with antibodies to Phenyl-N-Methyl-transferase and with immunohistochemical techniques, described a vasodepressor adrenalinergic system whose neurotransmitter was adrenaline. This "A-system" has conexions with hypothalamus, n. motor dorsalis of vagus nerve, n. tractus solitariis, l. ceruleus and the sympathic neurons of the lateral horn of the spinal cord. The fact that adrenaline is contained in regulatory cardiovascular nuclei above mentioned, suggest a role for adrenaline in the central regulation of blood pressure (De Jong et al. 1975; Saavedra et al. 1976; Scatton, Pelayo et al. 1979).
One of the most important explanations of central antihypertensor mechanisms of clonidine and of some active metabolites of alpha-Me-DOPA, such as alpha-Me-DA, and alpha-Me-NA is the action on $alpha_2$-adrenoceptors in CNS.
Recently Scatton, Pelayo et al. (1979) observed that clonidine decreases the turn-over and the potassium-evoked release of Adrenaline in brain areas involved in the regulation of blood pressure. This action may be mediated by the activation of inhibitory $alpha_2$-adrenoceptors located in noradrenaline neurons and/or on adrenaline cell bodies and nerve terminals. These results suggest that the "A-system" might also mediate the analgesic effects induced by clonidine.
Maggi et al. (1980) have studied alterations of sensitivity in brain membrane receptors labeled with ^3H-Dihydro-alprenolol (Beta), ^3H-WB-4101 ($alpha_1$) and ^3H-p.amino-clonidine ($alpha_2$). The results of these alterations indicate that long term activation of the beta-adrenergic system decreases the number of beta-receptors. This decrease rapidly and reversibly increases the number of $alpha_2$-receptors at central noradrenergic synapses. The beta-mediated regulation of $alpha_2$-receptor sensitivity in the brain may be a mechanism for the homeostatic control of central noradrenergic activity. The possibility of a mutual regulation between these two types of receptors could explain some central mechanisms of some antihypertensive drugs.
The existence of similarities between the cardiovascular effects of alpha-adrenoceptor agonists and opiate drugs is well known (Schmitt, 1976). Clonidine has been shown to produce hypotension by inhibiting the central sympathetic neurons. On the other hand morphinomimetic agents induce a decrease in sympathetic tone and an increase in vagal tone by an action on the medulla oblongata leading to hypotension and bradycardia (Laubie et al. 1974). Opiate receptors seem to be involved in these effects as nalorphine and naloxone prevented or reversed the hypotension and bradycardia induced by morphine-like drugs (Laubie et al. 1974).

2) According to Goldstein (1978) naloxone suppress: a) the food-reward reaction, b) the growth hormone release, c) the inhibition release of excitatory neurotransmitters, d) the analgesia by electrostimulation periaqueductal, and e) analgesia by acupuncture. All this implicates that endorphins have an important role in stress (Goldstein, 1978).
Faden and Holaday recently demonstrated that naloxone is able to reverse the hypotension of endotoxic-shock; this fact was explained because: 1) Endorphins are released in response to stress (Guillemin, 1978), 2) with very small doses of opiates are able to depress blood-pressure.
Experimentally in conscious rats naloxone in doses of 1 mg/kg rapidly increased the mean arterial pressure and pulse-rate in the shock by endotoxin.

Endorphins seem to have a most important role early in shock and naloxone was maximally effective in this time. Endovenously administered beta-endorphins produced a naloxone-reversible hypotension in conscious rats. The effects of naloxone in reversing the hypotension of both endotoxic and hypovolemic shocks, suggest that endorphins may be critical factors in other form of shocks (burn-shock, neurogenic-shock and so on). The fact that naloxone has been extensively used in humans in opiate overdose, along with its low toxicity and rapid onset of action, makes it a particularly atractive therapeutic agent (Faden and Holladay, 1979).

Chance and Schechter (1979) have studied the autoanalgesia phenomenon in which pain inhibitory mechanisms conditioned to fear, anxiety, shock, states of heroism, etc. are involved. In this mechanism the centrifugal inhibitory pathways interfere with the arrival of nociceptive impulses to the dorsal root neurons. As it is well known, when morphine was injected in PAG, a clear analgesic effect was produced which is mediated by centrifugal inhibitory fibers acting on neurons from dorsal root. This type of analgesia was blocked by phentolamine, a blocking agent of receptors $alpha_1$ and $alpha_2$; and by Methysergide, a 5HT-blocking agent. Thus, Nor-adrenergic and 5HT descending pathways are implicated in the analgesic effects of opiates. Yohimbine, a selective $alpha_2$ blocking agent, produced hyperalgesia and also blocked the autoanalgesia by a functional antagonism of descending triptaminergic and noradrenergic pathways.

Nevertheless, there is a lack of opiate antagonists in reversing autoanalgesia. Futhermore, although the final common descending pathways for opiate-analgesia and autoanalgesia appear to overlap or be identical, the cerebral events that activate these pathways may be different (Chance and Schechter). We think that there may be two different kinds of descending pathway conexions from PAG and closed brain areas (Takagi, 1980): a) to block or modulate the afferent nociceptive impulses on the dorsal horn (analgesia) and b) to modulate the efferent vegetative impulses to the sympathetic neurons in the lateral horn (cardiovascular tonus).

Arrigo-Reina and Ferry (1980) in rats exposed to a multiple-stress procedure (cold and fixation) exhibited a reduced paw oedema following an injection of carrageenin. Naloxone practically (5 mg/kg s.c.) did not significantly modify the intensity of the inflammatory reaction in non stressed rats, whereas it made the reaction fully evident in rats exposed to the stressful condition. Gastrical ulcerations were also increased with naloxone and were practically inexistent with 10 mg/kg s.c. of morphine. This investigation showed an increased intensity of the inflammatory response and gastric damage in stressed rats with the opiate-receptors blocked by naloxone. Therefore this fact supports the hypothesis of a role for endogenous opioid-peptides in the reactivity of the organism to stressful stimuli. In this regard Akil et al. 1980 have shown in rat brain that $ACTH_{1-24}$ displaces the tritiated beta-endorphin stereospecific binding in a dose dependent manner. A similar mechanism might take place at the stomach.

By Lewis et al (1980) has been observed that naloxone and dexamethasone both blocked analgesia for rats receiving prolonged, intermittent foot shock. These findings suggest that both opioid and nonopioid mechanisms underlie stress analgesia.

Severe stress cause a constellation of physiological changes (thermoregulatory, motor hormonal, respiratory and cardiovascular), some of which, like analgesia, have been antagonized by naloxone (Faden et al. 1979). The possibility that naloxone-sensitive stress analgesia may be secondary to one or more physiological effects cannot yet be dismissed.

3) The APUD-system (Amine Precursor Uptake Decarboxylation) or by other names such as paracrine, paraneuronal or peptidergic system has been defined by Pearse in 1969. The stores of this system contain both peptide hor-

mones and biogene amines, and further emphasize the close relationship between the endocrine and nervous system. In the APUD system all the active peptides concerned as much with CNS as with the peripheral-NS are included. These neuro-endocrine programmed cells originate in the ectoblast and make possible the coexistence of two neurotransmitters, in the same nerve terminals (Hokfelt et al. 1979 and 1980).

Evenwhen the immunohistochemistry technique has been very useful to visualize different types of compounds in nervous tissues, the problem is still very complex and cross-reactions with the antiserums are not uncommon and more than 20 peptides have been identified in brain neurons (Iversen, 1979). Larson (1979), provided evidence that Leu- and Met-enkephalins constitute a separate opiate peptide system and of course both are different from the beta-endorphins.

The peptides distribution is very complex. Some of them were possible to be recognized in: Primary sensory afferent spinal cord, Propio-spinal-interneurons, Central descending systems, Gastrointestinal tract, and in the Sympathetic ganglia.

According to Franz Gross (1980) and Ganten et al. (1980) some of the most important peptides participating on the regulation of the blood pressure are shown in the Table III.

TABLE III — PEPTIDERGIC SYSTEM

BLOOD PRESSURE REGULATION

I INCREASED CVT PNS & CNS	ANGIOTENSIN II LEU & MET-ENKEPHALIN (δ) ACTH & ADH
II DECREASED CVT PNS & CNS	SUBSTANCE P β-ENDORPHIN (μ)
III MIXED DECREASED CVT (PNS) INCREASED CVT (CNS)	KALIKREIN-KININ-SYSTEM

CVT = CARDIOVASCULAR TONUS
PNS = PERIPHERAL NERVOUS SYSTEM
CNS = CENTRAL NERVOUS SYSTEM

Angiotensine II increase the blood pressure in CNS by: a) increasing the sympathetic vascular tone, b) increasing the central vegetative discharges and c) producing the release of ADH and ACTH and the last one increases the release of Aldosterone and gluco-corticoids by the suprarenal gland.

One of the modern antihypertensor-drugs, the captopril, acts by blocking the synthesis of angiotensin II (it is a converting-enzyme inhibitor); by potentiating the peripheral pharmacodynamic effect of the BDK system and by inhibiting the degradation of the substance P.

From a pharmacodynamic point of view, there could be at least two opiate-peptide systems: μ-receptor-type for beta-endorphins and δ- receptor type for Leu and Met-enkephalins.

4) Several groups of authors have failed to obtain analgesia after intravenous or intracerebral microinjections of the enkephalins, Jacquet and Marks (1976) and Frenk et al. (1979) have reported analgesia in only 8 out of 19 rats with intravenous injections of 200 μg of Met-enkephalin.
Evidence from previous studies suggests that Met- and Leu-enkephalins induced analgesia (Belluzzi et al. 1977) as well as seizures (Frenk et al. 1979); both possibly interact with opiate receptors in the brain. These authors further demonstrated that such analgesic and epileptic effects are mediated by opiate-receptors in different brain areas: a) the ventromedial midbrain (PAG) and b) the dorsal medial thalamus, respectively, among possible others. Area a (PAG) is the most effective site for eliciting analgesia with microinjections of beta-endorphin, morphine, and electrical stimulation. In contrast, electrical stimulation in the rat of the thalamic dorsal medial nucleus (area b) gives rise to seizures, but not to real analgesia. Area (a) and (b) contain binding sites to enkephalins and beta endorphins (Simantov, et al. 1977).
The results of Frenk et al (1979) suggest that the opiate receptors mediating analgesia and seizures are not only located in different brain areas, but may also differenciate from one another pharmacologically. Lord, Watterfield, Hughes and Kosterlitz (1976) have presented the evidence indicating a heterogeneity of opiate-receptor types in the brain. Intravenous injections of enkephalins cause seizures at much lower doses than required for analgesia; whereas, similar injections of morphine cause seizures only at doses greatly exceeding the analgesic one. Frenk et al. (1978) suggest an interesting hypothesis: that enkephalin-induced seizures are mediated by δ-receptors in the dorsal medial-thalamus and enkephalin-induced analgesia is mediated by μ-receptors in the PAG. Enkephalins binding to the putative δ-receptors are more difficult to antagonize with naloxone than is their binding to the μ-receptors.
Cowan et al. (1979) administered several doses of different opiate compounds to rats 30 minutes before they were exposed to fluroethyl (Indoklon), a volatile convulsant drug. The time interval between the start of the infusion and the onset of a clonic convulsion was taken as the seizure threshold. Table IV shows the results obtained by Cowan et al. (1979), Frenk et al. (1979) and Kosterlitz (1978). Certain forms of human-epilepsy could be mediated by opiate-receptors in the forebrain. Therefore, the future discovery of a potent δ-antagonist might be expected to get a good therapeutic approach to an antiepileptic new drug.
It is worth noting that the physiological state of the CNS is the result of an opening and closing series on the interneuronal circuits whose consequences are the inhibition or the excitation processes. Besides, opioid-peptides and other factors, GABA has an important role in the inhibition processes of the CNS; Gale and Iadarola (1980), after studying some antiepileptic agents such as sodium valproate, support the concept that the anticonvulsant activity was only parallel to the ability to elevate GABA in nerve terminals.
When microiontophoresis opiate-drugs and opiate-peptides are applied in the majority of brain areas, a neuronal deppression in its functional activity was observed. This deppression was reversed by naloxone. A notable exception was registered at the hippocampus which is usually excited by opioids (but not always). This atypical excitation can be antagonized by: 1) iontophoresis of naloxone; 2) the GABA antagonist: bicuculine, or 3) by Mg^{++} salts. These results suggest that the opiates excite hippocampus pyramidal cells indirectly by inhibition of neighboring inhibitory interneurons. These inhibitory interneuronal phenomena are gabaergic since they are inhibited by opiate agents and are excited by bicuculine- that is, an excitation of hippocampus by desinhibition. This is explained by the local cytoarchitectonics or the desinhibition phenomenon and it seems to have some similari-

TABLE IV

OPIATE - RECEPTORS & EPILEPSY

EFFECTS	RECEPTORS	AGONISTS	NLX. TREAT.	LOCALIZATION
[A] ANTIEPILEPTIC	SIGMA(1)	SKF 10.047 / CYCLAZOCINE	NOT ATTENUATED	DOG SPINAL CORD
↑ CONVULSIVE THRESHOLD	μ	MORPHINE / ETORPHINE / B ENDORPH.	ATTENUATED	SP. CORD GUINEA-PIG-ILEUM PAG & BRAIN RAT
[B]	KAPPA	KETOCYCLA--ZOCINE.(KC) / ETHYL-KC. / NALORPHINE	NOT ATTENUATED	DOG SPINAL CORD
EPILEPTIC	?	MEPERIDINE / PENTAZOCINE	INCREASED	RAT BRAIN
↓ CONVULSIVE THRESHOLD	δ(2)	Leu-Enk / Met-Enk	BLOCKED BY HIGH DOSES	RAT BRAIN DORSAL-MEDIAL n. M. VAS-DEFERENS
	EMB(1)	MORPHINE AFTER NLX.		

(1) Not analgesic.
(2) 100 times less analgesic than μ.

Frenk et al.1978
Kosterlitz 1978
Cowan et al,1979

ty with the old "Hemmuns-Lämmung" of theGerman authors. The disinhibition phenomenon evoked by iontophoretic opioids could also underlie epileptiform effects of systemic administered opioids (Zieglgansberger et al. 1979).

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New Approaches to Antiviral Agents

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ABSTRACT

We are interested in two approaches to antiviral action. One of them is the antiviral activity of interferon and thus our studies on the molecular mechanism of action of human interferon on HeLa cells are described here. Furthermore we describe our work concerning the antiviral action of certain compounds which selectively enter virus-infected cells. Indeed the infection of animal cells by viruses leads to an increased membrane permeability in virus-infected cells. This increase in membrane permeability has allowed us to use selective inhibitors of virus-infected cells. Antibiotics like hygromycin B and anthelmycin that do not cross the cell membrane of normal cells, are able to penetrate into virus-infected animal cells, thereby inhibiting protein synthesis. The modification of membrane permeability is apparently rather non specific, since molecules with very different chemical structures cross the membrane after viral infection. Even macromolecules, such as alpha-sarcin, do also pass through the membrane of virus-infected cells. The modification of membrane permeability is brought about by a virion component, since it occurs very early during viral absorption to the cell, and is not blocked by inhibitors of gene expression.

KEYWORDS

Antiviral agents; animal viruses; protein synthesis inhibitors; ionophores; interferon.

INTRODUCTION

Very few compounds are available as useful antiviral agents (De Clercq 1979, Grunert, 1979) since the development of antiviral agents has been hampered mainly by two reasons. Firstly because viruses are intracellular parasites and for they replicative cycle use in most cases the same components as the cell does (Smith and Carrasco, 1978; Carrasco and Smith, 1980) and hence, there is not an obvious way to design selective agents that could specifically block virus replication, without affecting cell metabolism. Secondly our knowledge of the molecular biology of animal viruses is rather poor. A more profound knowledge of animal virus biology will provide indeed

with a rationale to design selective antiviral agents. Thus it is now well known that many viruses code for their own enzymes and in some cases no counterpart is found in the cell for such enzymes; this is the case of the reverse transcriptase (Verma, 1977; Chandra et al, 1979). In other cases the cellular and the viral enzymes differ in some characteristics in such a way that some inhibitors could preferentially block viral replication without causing a harmful effect on host metabolism. Such an approach has provided a number of compounds of potential value as antiviral agents (De Clercq, 1979). In the present work we describe that viral infection permeabilizes animal cells to a number of compounds, including translation inhibitors. Based on those findings a rationale is proposed to desing antiviral agents.

VIRUSES PERMEABILIZE CELLS DURING INFECTION

Throughout the development of an animal virus in its host cell there are interactions between some viral components and the cellular membrane. One can envisage at least four different ways during the viral life cycle in which the membrane could become modified: a) During virus attachment and absorption, b) by the insertion in the membrane of specific viral components, c) by modifying the metabolism or turnover of membrane components and d) at the time of the exit of the progeny virus.

Attachment of a number of enveloped and non-enveloped viruses produces changes in the fluidity of the lipids in the cytoplasmic membrane of the host cell (Levanon et al, 1977; Levanon and Kohn, 1978; Lyles and Landsberger, 1977 ; Moore et al, 1978). Indeed modification of membrane permeability during virus absorption has been well documented for Sendai viruses and a permeabilization of the cell to ions and low molecular weight compounds takes place. (Klemperer, 1960; Pasternak and Micklem, 1973, 1974; Impraim et al, 1980). At the same time the membrane potential drops to zero levels and as a consequence the transport of metabolites which is dependent on ionic gradients is inhibited, whereas those compounds that cross the cell membrane by a passive mechanism increase their content in the cell after viral infection (Imprain et al, 1980). Furthermore the modification of membrane permeability during picornavirus attachment is also well documented and in discussed in detail below. Viruses do also modify the structure of the membrane in their host cells by inserting some of their specific components. A large number of virus-specific surface antigens have been detected in virus-infected cells (Burns and Allison, 1977). In addition to the changes in membrane antigens, there are also modifications in the interaction of the cell surface with lectins (Nicolson, 1974). A redistribution of receptor and transplantation antigens on a variety of cells after viral infection has also been described. Evidence for the interaction of some viral components with the cell membrane during viral development is now overwhelming (Kohn, 1979; Carrasco and Smith, 1980). For instance, the G protein of rhabdoviruses is localized in the membrane of the cell during infection and the M protein is localized just beneath the plasma membrane and may be involved in the inhibition of the transport of several nucleosides (Genty, 1975). Cytocidal viruses also alter the lipid metabolism of their host. Picornavirus infection increases the synthesis of lipids and this phenomenon is accompanied by a great proliferation of intracellular membranes (Plagemann et al, 1970). A similar finding has been shown for adenoviruses (Mc Intosh et al, 1971) paramyxoviruses (Gilbert, 1963)

poxviruses (Gaush and Yougner, 1963) and papovaviruses (Norkin, 1977). The last step in the reproduction of viruses containing a lipid envelope is the budding through the cellular membrane. Budding also modifies the structure and the composition of the cell membranes.

INHIBITORS IMPERMEABLE TO NORMAL CELLS AS SELECTIVE ANTIVIRAL AGENTS

We have studied the modifications of membrane permeability after viral infection by means of translation inhibitors not permeable to normal cells in such a way that an increase of permeability is reflected in the passage of the inhibitor to the cell cytoplasm and this entry is quantitated by measuring the inhibition on protein synthesis in virus-infected cells (Carrasco, 1978, 1979).
The use of this technique has allowed us to detect modifications in membrane permeability at two different times during the viral life cycle of picornaviruses and possibly other animal viruses. One moment is when the virus attaches to the membrane and another time is later on in infection. Figure 1 shows that hygromycin B, an aminoglycoside antibiotic which does not pass through the membrane of normal cells, is able to inhibit protein synthesis in picornavirus-infected cells if it is present during the early times of infection. The inhibition is dependent on both the multiplicity of infection used and the concentration of hygromycin B present in the culture medium. The modification of membrane permeability early in infection seems to be a rather unspecific phenomenon as this specific inhibition of translation in virus-infected cells is observed with several compounds that possess different chemical structures, such as edeine, gougerotin, anthelmycin and hygromicin B.
The modification of membrane permeability is very accute in the late phase of infection at the time when big amounts of virus-coat proteins are synthesised (Carrasco, 1978, 1979; Contreras and Carrasco, 1979; Lacal et al, 1980). Again this modification in membrane permeability is rather non specific because different inhibitors and cellular metabolites are able to cross the cell membrane at this time of infection (for a review see Carrasco and Smith, 1980). The induction of the permeability change early in infection is brought about by a virion component, and takes place even in the absence of viral gene expression, whereas the apperance of membrane leakiness late is infection requires the replication and expression of the viral genome, at least when low multiplicities of infection are used. The degree of membrane leakiness all troughout the viral life cycle of EMC-infection of cells is shown in figure 2. The early membrane leakiness induced during viral attachment partially disappears in the next two hours after infection and reappears again at the time when the synthesis of viral proteins is apparent.
The modification of membrane permeability by animal viruses both early and late during infection is a rather widespread phenomenon, because is also observed with vesicular stomatitis virus (rhabdoviruses), adenoviruses, Semliki forest virus (togaviruses), Sendai virus (paramyxoviruses) and herpesviruses (Carrasco, 1978; Contreras and Carrasco, 1979; Lacal et al, 1980; Benedetto et al, 1980). The modification of membrane permeability to hygromycin B during the absorption of vesicular stomatitis virus is demostrated by the experiment shown in figure 3.
To investigate whether the induction of membrane permeability by viruses can be used as an approach to design antiviral agents, we tested the inhibition of virus yield in the presence of hygromycin B both when the antibiotic was present during the early period of

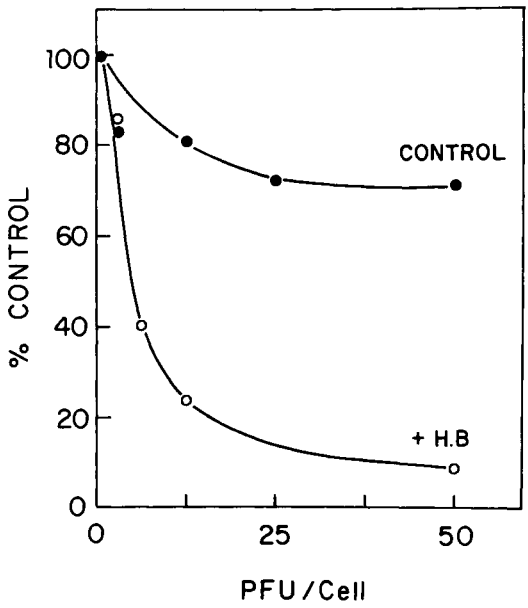

Fig. 1. Effect of EMC infection of mouse L929 cells on membrane permeability. 2 mM hygromycin B was present during the first hour of virus absorption (o—o). Protein synthesis was estimated from 1-2 hours of the viral replicative cycle and the percentage of the control is represented.

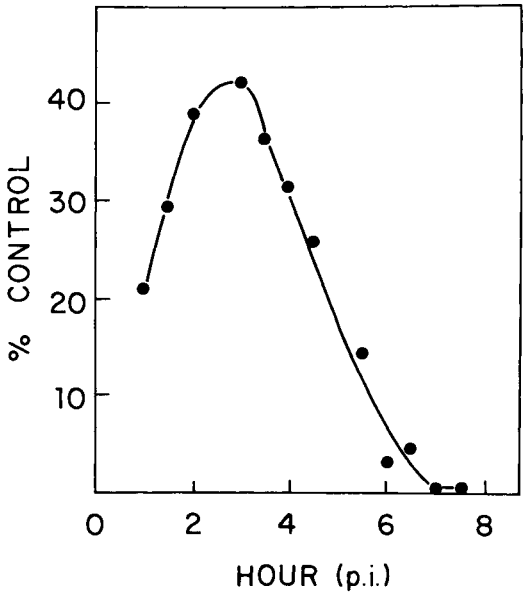

Fig. 2. Membrane leakiness throughout the EMC reproductive cycle in L929 cells. Membrane leakiness was estimated by means of hygromycin B. The multiplicity of infection used was 30 PFU/cell. Protein synthesis was measured every half hour in the absence or in the presence of 10 mM hygromycin B and the percentage of the control is represented.

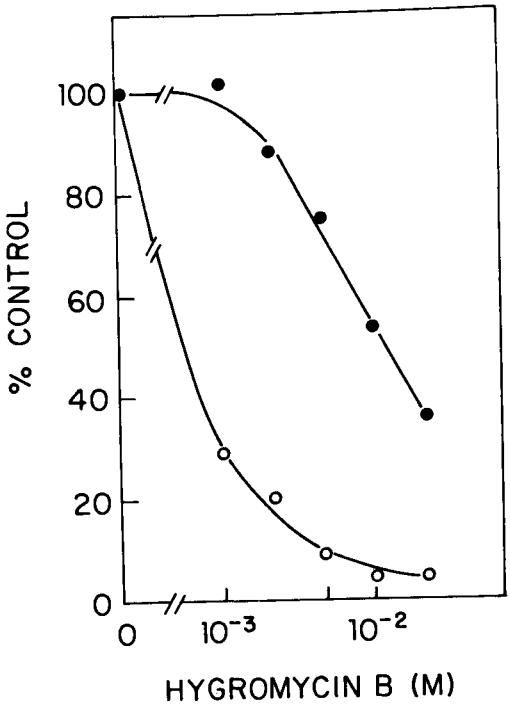

Fig. 3. Effect of vesicular stomatitis virus infection of L929 cells on membrane permeability. The multiplicity of infection used was 100 PFU/cell. Different concentrations of hygromycin B were present during the first hour of viral attachment. Protein synthesis was measured in the second hour of the viral life cycle. (●—●) Control; (o—o) plus vesicular stomatitis virus.

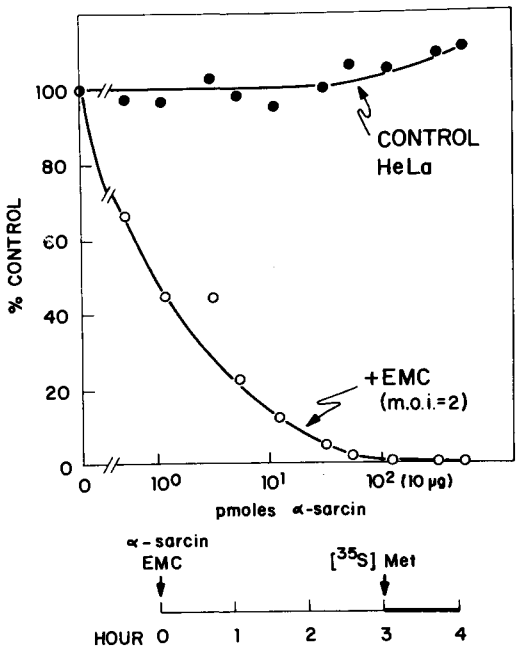

Fig. 4. Effect of EMC infection on membrane permeability to alpha-sarcin. Different concentrations of the plant toxin alpha-sarcin were present during the first hour of EMC absorption. Protein synthesis was measured from 3-4 hours as indicated.

infection (Table 1) and when the antibiotic was present at later limes of the viral life cycle (Lacal et al, 1980; Benedetto et al, 1980). In both instances the presence of hygromycin B resulted in a significant inhibition of virus yield in a single step growth cycle.

TABLE 1. Inhibition of EMC production by hygromycin B.

Hygromycin B (M)	% Control
--	100
10^{-3}	2,7
10^{-4}	45

Hygromycin B was present during the first hour of the viral replication cycle. Afterwards, the medium was replaced by fresh medium and the virus was collected and titrated after 24 hours.

PROTEIN TOXINS CAN PENETRATE INTO VIRUS-INFECTED CELLS.

The experiments discussed in the previous section indicated that low molecular weight compounds, such as hygromycin B or nucleotide analogs, cross the membrane of virus-infected cells rather freely. Our early idea was, that compounds with a molecular weight over 750-1000 were too big to cross the membrane of a virus-infected cell. However, this idea has now been modified by new experimental evidence obtained with protein toxins.

There are a number of protein toxins that inhibit protein synthesis in eukaryotic cells (Vázquez, 1979). The mechanism of action of these inhibitors, like ricin and abrin, involves two steps: The recognition of a cell surface receptor and subsequent attachment of the toxin to the membrane, followed by the entrance of the toxin moiety, so called effectomer, or A chain. Once in the cytoplasm this effectomer molecule blocks protein synthesis by inactivating enzimatically the ribosomes (Olsnes et al, 1975).

The simultaneous presence of both chains of these toxins powerfully inhibits protein synthesis in intact cells, whereas the isolated A chain does not show such activity. On the other hand inhibition of translation in cell-free systems is achieved by the isolated A chain (Fernández-Puentes and Carrasco, 1980). We have compared the activity of several toxins including abrin A chain alpha-sarcin, mitogillin, restrictocin and PAP which appear to be analogous to abrin A chain (Fernández-Puentes and Carrasco, 1980). All of them showed a preferential inhibition of protein synthesis in virus-infected cells, but alpha-sarcin was the most powerful inhibitor and showed the highest selectivity (Figure 4). These result are interpreted to mean that viral entry into the cell also promotes the entry of the toxin, which otherwise is unable to penetrate into normal cells. The molecular mechanism underlying this phenomenon is not yet clear. However, the possibility that the toxin binds to the virus and uses the virion particles as carriers to reach the cell cytoplasm is not supported by our experiments. Thus figure 5 shows that no binding between the toxin and the virion particles can be detected. Moreover, addition of the toxin once the virus has passed the cell membrane, also results in an inhibition of virus-infected cells (Fernández-Puentes and Carrasco, 1980). That is, even in the absence of virus-particles in the medium the toxin crosses

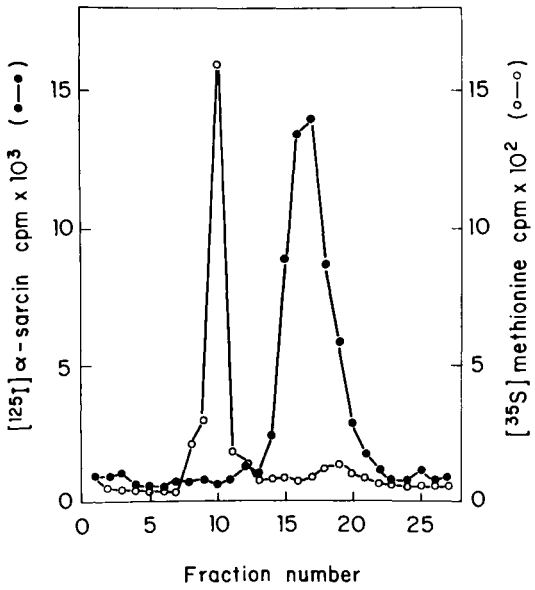

Fig. 5. Sepharose 6B column chromatography of (35S)-methionine labelled EMC virus (o—o) and (125I) labelled alpha-sarcin (•—•).

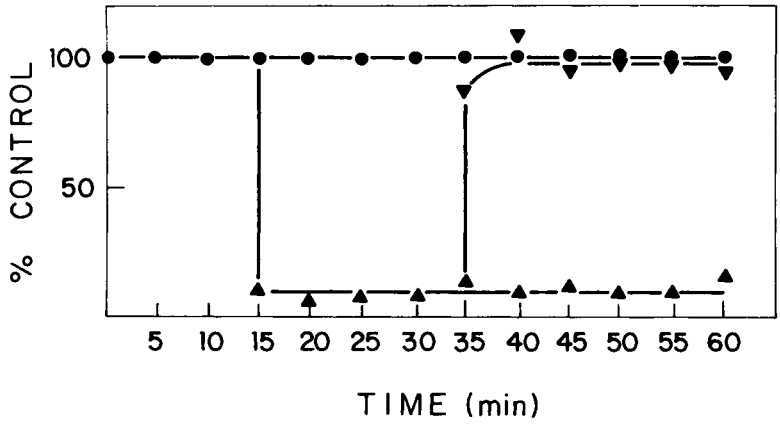

Fig. 6. Inhibition of protein synthesis in HeLa cells by nigericin. Protein synthesis was measured with (35S)methionine every 5 minutes. (•—•)Control; (▲—▲) plus 2.10^{-6} M nigericin. After 35 minutes incubation, the medium was replaced by medium containing 50 mM KCl and 25 mM NaCl. 10 µg/ml actinomycin D were present throughout the experiment.

the cell membrane if it has been previously permeabilized by viral absorption. Once again, this permeability change is not specific for picornaviruses, and is also observed after togavirus or adenovirus infection (Fernández-Puentes and Carrasco, 1980). These proteins show a marked antiviral activity in cell culture and are not toxic to cells at concentrations that prevent virus replication.

THE MODIFICATION OF MEMBRANE PERMEABILITY BY ANIMAL VIRUSES AND IONOPHORES IS SIMILAR.

Once we observed the modification in membrane permeability brought about by viral infection, it was of interest to know the molecular mechanisms that the virus uses to permeabilize the cells. It is also of interest to know the consequences for cellular metabolism of such a modification of the cell membrane.
One approach used to investigate these problems was by means of membrane active compounds (Pressman, 1976). The rationale behind this approach is to stablish it there is a similarity between a simple model system like an ionophore and the modifications observed in the cell after viral infection. If this is so,the enormeous amount of information on the molecular mechanism of ionophore action, will facilitate the understanding of viral action on the membrane. First of all we wanted to know whether the action of a membrane-active compound could block translation and whether this inhibition was mediated by monovalent ions. Figure 6 indicates that the addition of nigericin to human HeLa cells inmediatelly stops protein synthesis, but interestingly enough this inhibition is totally reversed by the ionic modification of the medium. In this respect the ionophore behaves as a virus because they both inhibit translation in the infected cell and this inhibition is reversed by the modification of the ionic composition in the outside medium (Alonso et al, 1979; Alonso and Carrasco,unpublished observations).
The second question was whether nigericin was also able to permeabilize the cells to inhibitors of protein synthesis not permeable to normal cells,as viruses do. Figure 7 indicates that indeed this is the case and at concentrations of nigericin that are not harmful for translation, the cell becomes permeabilized to hygromycin B (Alonso and Carrasco, 1980). This is another piece of evidence in favor of the paralelism between ionophore action and viral infection.
Moreover, as we discussed in the previous section the absorption of virion particles to cells permeabilize them to protein toxins. Figure 8 shows that nigericin treatment of cells makes the cell membrane also permeable to alpha-sarcin. Therefore, these experiments indicate that "impermeable inhibitors" of translation can be introduced into cells by means of either ionophores or virion particles.

ANTIVIRAL PROPERTIES OF INTERFERON

Interferons are inducible glycoproteins synthesised by a variety of vertebrate cells in response to a number of external stimuli like, for instance, viral infection (Friedman, 1977). Treatment of a cell with interferon produced by a cell of the same species, renders the cell resistant to viral infection. It is accepted that interferon treatment induces an antiviral state in the cell, for which gene expression is necessary, but the exact molecular mechanisms underlying this antiviral state, are still little understood. Cell-free systems obtained from interferon treated cells are more susceptible to be inhibited by dsRNA (Baglioni, 1979; review). This result has been atributed to an increased phosphorilation activity that would

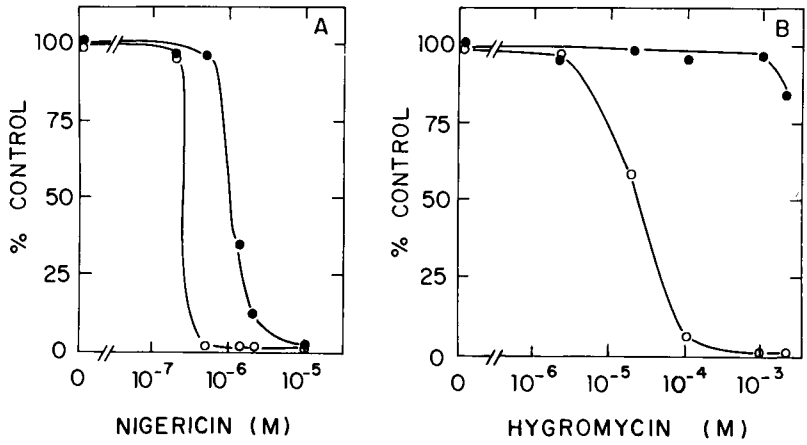

Fig. 7. Permeabilization of HeLa cells to hygromycin B by nigericin. Hygromycin B and nigericin were added at 0 time. The labelling period to estimate protein synthesis was from 4 to 5 hours. Panel A: (●—●) nigericin; (o—o) nigericin + 10^{-3}M hygromycin B. Panel B: (●—●) hygromycin B; (o—o) hygromycin B + 10^{-7}M nigericin.

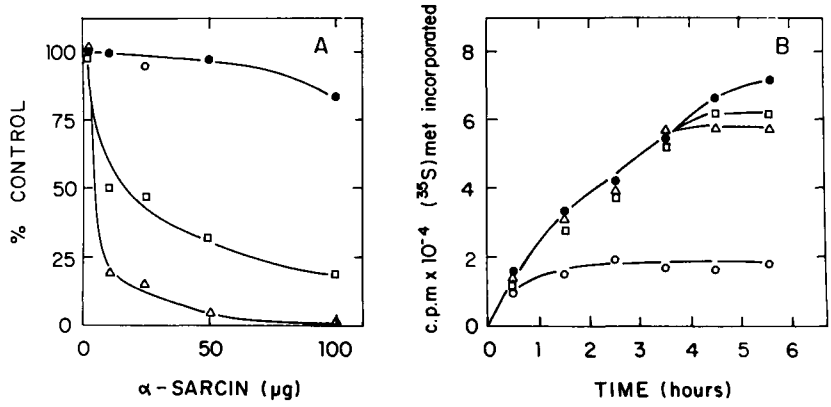

Fig. 8. Permeabilization of HeLa cells to alpha-sarcin by nigericin. Labelling was as indicated in figure 7. Panel A: (●—●) alpha-sarcin; (△—△) alpha-sarcin + 6.10^{-7}M nigericin; (□—□) alpha-sarcin + 4.10^{-7}M nigericin. Panel B: (●—●) Control; (△--△) 6.10^{-7}M nigericin; (□—□) 50 μg/0.5 ml alpha-sarcin; (o—o) 6.10^{-7}M nigericin + 50 μg alpha-sarcin.

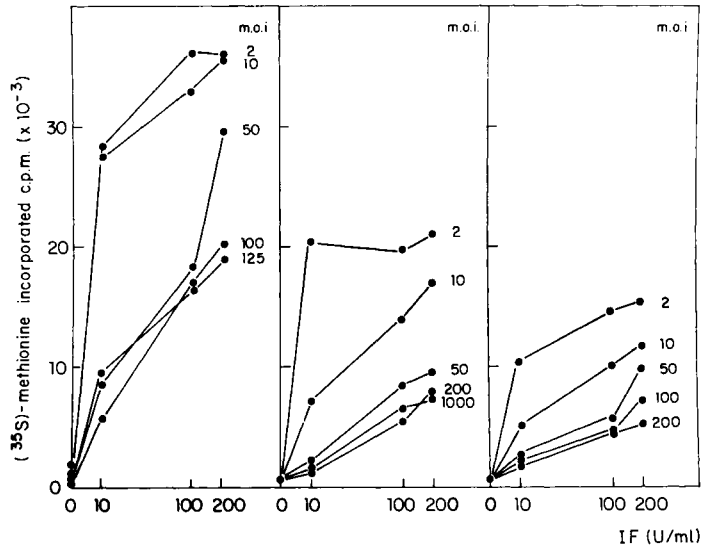

Fig. 9. Effect of different concentrations of interferon and EMC virus on cell survival. HeLa cells were incubated overnight with the indicated amounts of human interferon and infected with EMC at the indicated multiplicity of infection. Protein synthesis was estimated one day after virus infection.

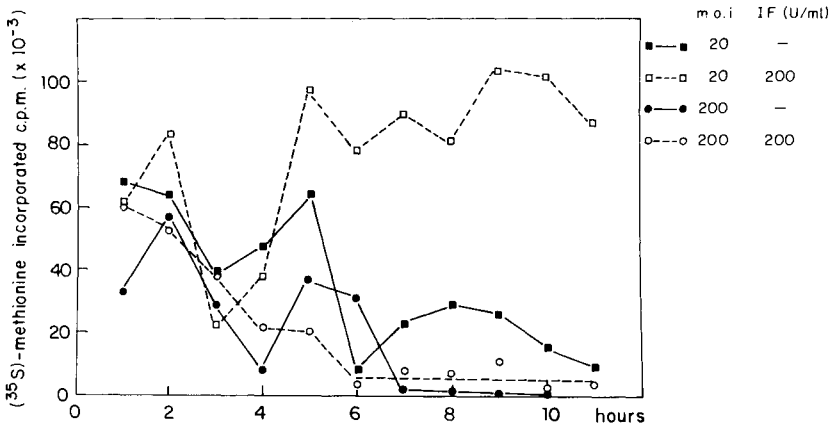

Fig. 10. Time course of protein synthesis in EMC-infected HeLa cells. Effect of human interferon.

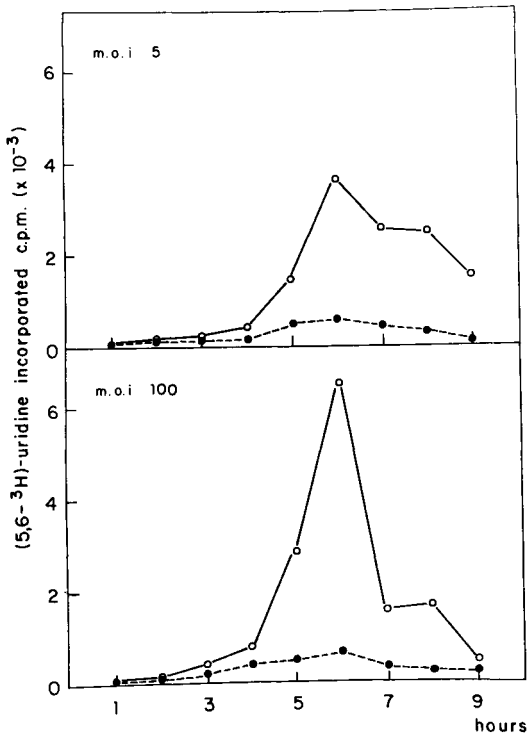

Fig. 11. Effect of interferon treatment on viral RNA synthesis in HeLa cells infected with EMC virus. (o—o) Control; (●—●) plus IFN.

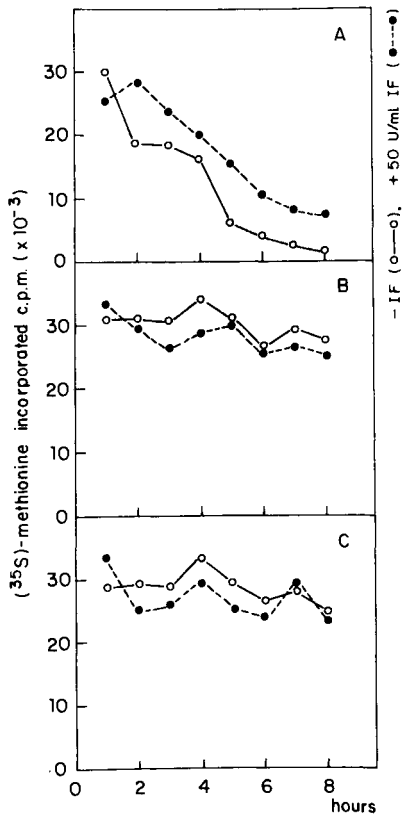

Fig. 12. Effect of UV-inactivated EMC virus on protein synthesis in interferon-treated HeLa cells. Panel A: Control; Panel B: UV-inactivated EMC virus. Panel C: heat-inactivated EMC virus.

inactivate some components of the protein synthesising machinery and also to an increased nuclease activity (Revel and Groner, 1978; Baglioni, 1979). An enzyme that in vitro synthesises (2'-5')oligo A from ATP is induced after interferon treatment, this enzyme is activated by dsRNA and the final product of the reaction, the so-called 2-5A, activates a nuclease that degrades both viral and cellular mRNA (Williams and Merr, 1980). Evidence from interferon-treated virus-infected cells has questioned the involvement of the phosphorilation mechanism in vivo (Gupta, 1979) and as yet no experimental evidence is available to indicate whether the nuclease system operates in the cell.

We have analysed the translation capacity of HeLa cells infected by EMC virus, after the induction of the antiviral state by treatment with human limfoblast interferon, generously given to us by Drs. Finter, Fantes and Johnston (the Wellcome research laboratories). It is not clear from the literature whether interferon treatment and subsequent infection of cells, leads to cell death, or whether the cell survives viral infection, or whether the cell recovers and continues synthesising proteins at control levels after a transient period in which the cell stops making proteins (Falcoff and Sanceau, 1979). The experiment shown in figure 9 indicates that human interferon treatment and subsequent infection of HeLa cells does not result in an all or none protection. Under low multiplicities of infection and high interferon concentrations the cell survives, whereas the increase of viruses per cell during infection and the decrease of interferon in the culture medium leads to cell death after EMC infection. However, in all these situations, viral reproduction is strongly inhibited. Figure 10 shows the time course of protein synthesis after EMC-infection when low and high multiplicities of infection are used. Analysis in polyacrylamide gels of the proteins synthesised under both situations indicates that no viral proteins are observed, even though cellular protein synthesis is strongly inhibited by high multiplicities of infection. As indicated above, if a high m.o.i. of virus is used, the cell does not recover even after 30 hours of infection and after repeated addition of interferon after infection. The synthesis of viral RNA is strongly inhibited (figure 11), perhaps as a consequence of the inhibition of viral protein synthesis, that will result in a lack of synthesis of the viral replicase.

As shown in figure 10 there is a strong shut-off of host protein synthesis in interferon-treated cells when a high m.o.i. of virus is used. We wanted to know whether for this shut-off to occur, viral gene expression was necessary. The results shown in figure 12 indicate that U.V-inactivated EMC virus does not induce the shut-off of translation in interferon-treated cells, indicating that gene expression is necessary for this phenomenon. Work is now in progress to investigate the molecular mechanism underlying the impairment of EMC replication in interferon treated HeLa cells and the mechanism by which protein synthesis is shut-off in those cells.

CONCLUDING REMARKS

The observation that virus infection permeabilizes animal cells to a number of inhibitors of translation has allowed us to use these compounds as selective agents that block protein synthesis in virus-infected cells. According to our latest results there is not a limit in the molecular weight of the inhibitor to cross the cell membrane of virus-infected cells, because a selective inhibition is achieved

with both high molecular weight compounds, such as alpha-sarcin and low molecular weight compounds, like hygromycin B. although these inhibitors show a marked antiviral activity in cultured cells, it still remains to be stablished their usefullness as antiviral agents in whole organisms.

ACKNOWLEDGEMENTS

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New Antiviral Agents: Some Recent Developments

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ABSTRACT

A brief review is presented of recent progress in the development of antiviral agents, with particular emphasis on those with efficacity against influenza, the common cold, and various herpes viruses. The mechanism(s) of action of some of the compounds are described, including the role of virus-coded proteins and enzymes.

KEYWORDS

Viral diseases; antiviral chemotherapy; design of antiviral agents; respiratory viruses; herpes viruses; infections agents; chemical syntheses; structure-activity relationships; virus-coded enzymes and proteins.

INTRODUCTION

Antiviral chemotherapy has, in the past 3-5 years, undoubtedly turned the corner, and come of age. It should, nonetheless, be noted that the presently effective, or potentially effective. agents have been largely the outcome of random screening procedures. The role of the medicinal chemist has been to apply synthetic methods to modification of compounds with established activity (structure-activity relationships) to produce analogues with enhanced activity, reduced toxicity, improved ability to traverse cell membranes or the blood-brain barrier, etc. This esthetically unsatisfying, albeit frequently productive, situation is undergoing some improvement. The remarkable advances in the molecular biology of the replicative processes of viruses have led to pin-pointing a number of targets of potential value in chemotherapy, e.g. virus-specified macromolecules such as nucleic acids and proteins, the latter including enzymes with specificities differing from those of the host cell (see extensive review by Kit, 1979). Several of these enzymes, like nucleoside and nucleotide kinase, determine the effectiveness of some agents (Chen and Prusoff, 1978; Fyfe and others, 1978). Others, such as polymerases, have led to intensive searches for specific inhibitors (see below). The attractiveness of the infectious component, nucleic acid, as a target site accounts for the extensive research on nucleoside and nucleotide analogues.

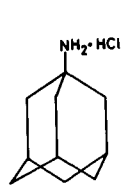
FACTORS IN DEVELOPMENT OF ANTIVIRAL AGENTS

The goal of the medicinal chemist in an academic or institutional environment is to

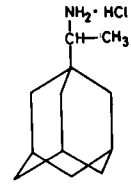
produce an antiviral agent at the laboratory level. The subsequent road to clinical trials is an intricate process, dependent on numerous factors such as range of applicability, effectiveness of laboratory models to predict efficacy in humans, the increasing complexity of safety regulations, and, not least important, economic factors. The pharmaceutical industry is the ultimate producer, and the cost of bringing a new antiviral drug from the lab bench to the clinic is probably no less than for other drugs. This accounts in part for the emphasis on development of agents against respiratory and herpes viruses. The practical aspects involved in the development of antiviral drugs have been admirably reviewed by Bucknall and Rutty (1977) and Grunert (1979), with special emphasis on the choice of laboratory and animal models of virus diseases. It is rather odd that so little effort has been devoted to a search for agents active against pathogenic viruses in domestic animals, notwithstanding its economic and clinical relevance; such research would be expected, in turn, to provide useful information to carry over into the field of antiviral agents for use in humans. Furthermore, there are instances where the nature of the infectious agents remain to be established, e.g. scrapie in sheep, or the related Creutzfeld-Jacob disease and Kuru in humans, all three of which, by application of target theory to their inactivation by ionizing radiations, appear to be of the size of plant viroids (Gibbs and others, 1978), hence for the moment intractable to any approach other than random screening. Finally, it is most satisfactory to note the increasing emphasis on quantitative data both at the laboratory level, and on tests in humans; the very fact that double-blind placebo-controlled trials are frequently required testifies to the complexities involved in introducing an antiviral drug to the clinic.

ADAMANTANE ANALOGUES

Two candidates against influenza have hitherto been subjected to controlled clinical trials, amantadine and ribavirin. Amantadine·HCl, or 1-aminoadamantane·HCl,



Amantadine·HCl



Rimantidine·HCl

a cyclic primary amine, is by no means a new antiviral drug. Of many compounds tested, it is the parent of a class unequivocally useful against influenza in man. First uncovered during randon screening in 1963, it has had a chequered career with 15 years controversy regarding its efficacity in prophylaxis and chemotherapy of influenza A infections, although licensed initially in the USA in 1966 (but only for use against the Asian H2N2 strain), and subsequently against all A strains. It has been widely tested and employed in the USSR (Smorodintsev and others, 1970a, 1970b; Oxford and Galbraith, 1980).

What is new, and of general significance for antiviral chemotherapy, is the November, 1979, decision of an NIH panel to recommend widespread use of amantadine for prophylaxis and therapy of all strains of influenza A (the drug is inactive against B strains). The necessity was also underlined of accelerating studies on the numerous known amantadine analogues, particularly rimantidine (∝-methyl-1-adamantane methylamine), reported more active, better tolerated in humans, and with a broader antiviral spectrum.

Although extensively investigated, the mechanism of action of amantadine is not

fully elucidated. There is some evidence suggesting interference with the process of penetration through the cell membrane. But, under conditions where penetration occurs (established by electron microscopy), there is no synthesis of virus specific RNA or polypeptides, and there is general agreement that its site of action is at the stage of viral uncoating, or shortly thereafter (Oxford, 1980). Oddly enough, it is not known whether the drug itself is metabolized. Further research is obviously called for, and will certainly be stimulated by the increased acceptance of the drug.

RIBAVIRIN

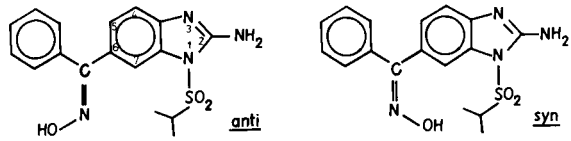
Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), originally designated as "virazole", is a potent *in vitro* inhibitor of a broad range of RNA and DNA viruses. Particularly striking are its reported inhibitory effects against influenza and parainfluenza virus replication in cell and organ cultures, and in some animals. Although licensed for human use in some countries, and undergoing clinical trials in others, reports on its efficacy are conflicting. But its broad spectrum *in vitro* is alone sufficient to warrant more detailed studies on mechanism(s) of action. Structure-activity investigations underline its specificity (Sidwell and others, 1979; Drabikowska and others, 1979).

The nucleoside is converted intracellularly by adenosine kinase (Willis and others, 1978) to the 5'-phosphate, a potent competitive inhibitor of IMP dehydrogenase, thus inhibiting conversion of IMP to XMP, the immediate precursor of GMP (Sidwell and others, 1979). This may account in part for both the cytostatic and broad-spectrum antiviral effects, reversible by guanosine and xanthosine, but this has been questioned (Oxford, 1975; Scholtissek, 1976). The subsequent finding that the nucleoside is converted intracellularly to the 5'-triphosphate (Streeter and others, 1977), which selectively inhibits influenza virus RNA polymerase (Oberg and Helgstrand, 1977) relative to a number of other RNA or DNA polymerases (Eriksson and others, 1977; Muller and others, 1977), supported an earlier proposal that the activity of ribavirin against influenza may reside in inhibition of RNA synthesis (Scholitissek, 1976).

More recently, noting that ribavirin is inactive against polio, the RNA of which does not contain a 5'-terminal methylated "cap", it was shown that ribavirin-5'-triphosphate is a potent competitive inhibitor of vaccinia mRNA:guanyltransferase, thus preventing capping 5'-guanylation of viral mRNA (Goswami and others, 1979); only higher concentrations inhibited cap methylation in absence of GTP. However, similar inhibitory effects prevailed when the substrate was uncapped rat liver mRNA, raising some question regarding specificity towards viral mRNA. In yet another study, Sarver and Stollar (1978) found that ribavirin inhibition of the cytopathic effect of Sindbis virus against an LF-C-7 clone of *Aedes albopictus* cells appeared to involve interference with some cell function(s) essential for a late step in assembly of mature infectious particles. Finally, 3-amino-1,2,4-triazole, a close analogue of the ribavirin aglycone (1,2,4-triazole-3-carboxamide), a herbicide and inhibitor of heme synthesis in rat liver, inhibits the growth of *Neurospora crassa* and produces biochemical changes identical to those provoked by chloramphenicol, i.e. directly and specifically inhibiting protein synthesis on mitochondrial ribosomes (Kumar and Padmanaban, 1980). Since the aglycone of ribavirin also exhibits some activity, assumed due to its conversion by purine nucleoside phosphorylase to the nucleoside (Streeter and others, 1977), it is conceivable that the aglycone may act at the level of inhibition of protein synthesis, particularly in view of the cytostatic effects of the nucleoside on L5178y cells (Muller and others, 1977). Ribavirin itself has been reported to selectively inhibit polypeptide synthesis induced by influenza A and B viruses in treated cell monolayers (Oxford, 1975). Curiously, it is not known whether ribavirin is incoporated into nucleic acids.

BENZIMIDAZOLE ANALOGUES

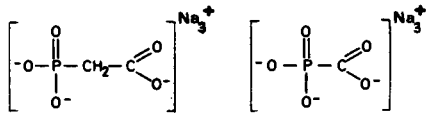
Initial reports, over 30 years ago, of antiviral activity of benzimidazole stimulated the synthesis and evaluation of a large number of analogues, one of the better known being 2-(α-hydroxybenzyl)benzimidazole, shown to be a specific inhibitor of picornaviruses (Eggers and Tamm, 1966) from its ability to induce or select resistant mutants (see Hermann and Hermann, 1977). A current concerted effort by DeLong's group at Eli Lilly has turned up the syn and anti isomers of 6-[{(hydro-



xyimine)phenyl}methyl]-1-[(-methylethyl)sulfonyl]-1H-benzimidazol-2-amine (Wikel and others, 1980). Their significance resides in their activity against all picornaviruses tested and, in particular, against all 43 of 43 rhinovirus types (with the anti isomer the more active). The latter result is of special interest because of difficulties associated with clinical diagnosis of type and strain of rhinoviruses, and attendant difficulties in developing a vaccine against the common cold. Tests on blood and lung levels in mice and dogs, and activity in rhinovirus-infected human organ cultures, also appear promising; but there is a sparsity of data on therapeutic indexes. Apparently structure-activity studies have been extensive, since proper substitution at positions 1, 2 and 6 of the benzimidazole ring are essential for activity. Bearing in mind the key importance of candidates against respiratory viruses, further developments will be awaited with interest.

A number of benzimidazole nucleosides, especially halogenated derivatives, are reported active (reviewed by Sehgal and Tamm, 1968; but see Diwan and others, 1968), but does not appear to have aroused much interest. This is surprising in the light of the fact that the 5,6-dichloro derivative of benzimidazole riboside is a specific and reversible inhibitor of nuclear hnRNA synthesis (Egyhazi, 1974; Sehgal and Tamm, 1978) and is widely employed as a tool in studies on RNA transcription in cellular and viral systems; it is also a "superinducer" of interferon production (Sehgal and Tamm, 1978). Little is known about the mechanism(s) of these processes, but structure-activity relationships on inhibition of transcription are under investigation (Egyhazi and Shugar, 1979; Kazimierczuk and others, 1980).

PHOSPHONOACETATE (PAA) AND PHOSPHONOFORMATE (PFA)



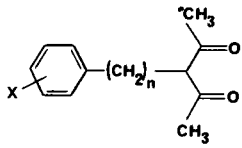
PAA, synthesized by Nielen in 1924, turned up in 1973 during a random screen as an effective agent against herpes virus infections in mice, and has been intensively investigated (reviewed by Boezi, 1979). Structure-activity studies led to new analogues, of which the most promising is PFA (Helgstrand and others, 1978). The specificity of the foregoing, which may be considered pyrophosphate analogues, and active against almost the entire herpes group, is ascribed to selective inhibition of HSV-coded DNA polymerases by interaction with the latter at the pyrophosphate

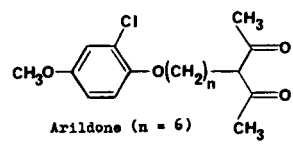
binding site. Inhibition by PAA is non-competitive with regard to both substrate and template, with K_i as low as 1 μM. Cellular DNA polymerase α is also affected, but K_i is 15 to 30-fold higher, whereas polymerases β and γ, and a variety of other prokaryotic and viral polymerases are insensitive (Erikkson and others, 1980). PAA weakly inhibits vaccinia replication and, in accordance with this, is a weak inhibitor of vaccinia polymerase. The drug itself is apparently not metabolized. PAA is as effective as, in some instances more so than, araA or araAMP, in treatment of herpes infections in animals. But toxicity problems associated with its use in systemic infections, pronounced dermal toxicity when used topically, and tendency to accumulate in the bone (the possible toxicity of which has not been evaluated), make it an unlikely candidate for clinical trials.

PFA, also retained in the bone, does not exhibit dermal toxicity, and appears a promising candidate for topical treatment of HSV infections, including ocular, skin and genital lesions. Results of clinical trials, under way, will be awaited with interest. PFA also shows promise as a candidate for therapy of hepatitis B, in that it (but not PAA) effectively inhibits Dane particle DNA polymerase (Nordenfelt and others, 1979). It also inhibits cytomegalovirus replication at non-toxic concentrations (Wahren and Oberg, 1980). Its possible use in systemic herpes infections will depend on whether its accumulation in the bone is indeed harmful.

ACYCLIC β-DIKETONES

A significant recent development is the finding, during routine screening, that a number of aryl alkyl diketones of the general structure shown, exhibited *in vitro* activity *vs* equine rhinoviruses. Subsequent extensive structure-activity relationships turned up a variety of analogues with significant activities against a number of RNA and DNA viruses at concentrations in the uM range (Diana and others, 1978, and references cited). The most promising, 4-[6-(2-chloro-4-methoxyphenoxy) hexyl]-3,5-heptanedione (Arildone), inhibits the *in vitro* cytopathic effects of HSV-1, HSV-2, VZV and a variety of RNA viruses, e.g. poliovirus type 2 replication





Arildone (n = 6)

in HeLa cells was affected at a minimal inhibitory concentration of 0.25 uM. At concentrations 10-fold higher, there was no effect on cellular protein, RNA or DNA synthesis. Other uninfected cell types were equally, or more, refractory to the drug. *In vivo* studies have shown Arildone to be effective against HSV-1 and HSV-2 guinea pig skin infections (G. Diana, personal communication).

The drug is not virucidal. It inhibits uncoating, but not adsorption or penetration (McSharry and others, 1979). Hence, like rhodanine (Eggers, 1977), it blocks virus-induced shut-off of host cell protein synthesis without interfering with cell functions. But the structure, and antiviral spectrum, differ from those of rhodanine. Preliminary results suggest a similar mechanism of action against HSV (Kuhrt and others, 1979). Arildone may produce changes in the viral capsid sufficient to interfere with, or to prevent, interaction of the virion with cellular membranes during uncoating, in accordance with structure-activity studies, which suggest that lipophilic substituents on the phenyl ring are essential for activity. The lipophilic nature of the drug may also account for its inhibition of thymidine and uridine transport across the plasma membrane at high drug concentrations.

GLUCOSE ANALOGUES AND GLUCOSIDES

Since the report in 1959 by Kilbourne on the activity of 2-deoxy-D-glucose against influenza virus, a number of such analogues with in vitro antiviral activities has been reported, including glucosamine, 2-deoxy-2-fluoro-D-glucose, 2-deoxy-2-fluoro-D-mannose, tunicamycin, all active against enveloped viruses (Scholtissek, 1975; Shannon and Schabel, 1980). This culminated in a report on the succesful treatment of human genital herpes infections in 36 women, in a double-blind, placebo-controlled study (Blough and Giuntoli, 1979). The results of this latter study have been questioned (Corey and Holmes, 1980), while Shannon and Schabel (1980), stimulated by the foregoing, conducted analogous trials on HSV-2 genital infections in guinea pigs, with negative results. Additional trials will obviously be required to define the clinical utility of deoxyglucose (and perhaps other analogues), simplified by the fact that the compound exhibits relatively low toxicity, and readily penetrates the cell.

The foregoing glucoside analogues are known to inhibit viral replication by interfering with glycosylation of virus-coded glycoproteins which are constituents of the viral envelope. In fact, these inhibitors of protein glycosylation in general were initially discovered by virtue of their antiviral activities (Schwarz and Datema, 1980; Parodi and Leloir, 1979). Recent studies have shown that 2-deoxy-D-glucose, as an analogue of glucose and mannose, is converted intracellularly to the UDP- and GDP- derivatives, which inhibit glycosylation; GDP-deoxyglucose appears to be mainly responsible for the inhibition, since addition of mannose, leading to reversal of inhibition of virus formation, reduces the level of GDP-deoxyglucose, but not UDP-deoxyglucose. In the presence of one of these inhibitors, virus particles are formed containing glycoproteins with decreased amounts of carbohydrate, by interference with transfer of complete oligosaccharides from a precursor to the viral proteins (Schwarz and Datema, 1980, and references therein).

A recent development which, if confirmed, would be of remarkable significance, is the demonstration that some phenyl glucosides exhibit potent virucidal effects specifically against enveloped viruses (Sugita and others, 1979a,b). Structure-activity studies pointed to p-alkylphenyl 6-halogeno-6-deoxy-β-D-glucosides as the most effective, the best member being the p-(sec.-butyl)phenyl derivative, which was claimed to be 70-fold more active (at 0.03 mM) than 2-deoxy-D-glucose against HSV and influenza. At a concentration of 0.1 mM there was a 3 log decrease of influenza virus multiplication on LLCMK$_2$ cells. There was no activity against polio, which has no envelope. Activity against paramyxoviruses, but not myxoviruses and other enveloped and non-enveloped viruses, indicated that the mechanism of action of these analogues differs from that of 2-deoxy-D-glucose and glucoseamine. More detailed studies with Sendai virus (Fushimi strain) demonstrated that loss of infectivity was accompanied by a concomitant decrease in neuraminidase, haemegglutinating and hemolytic activities. The comparable decreases in neuraminidase and haemagglutinating activities indicate that the inhibitor may affect the biosynthesis of the viral glycoprotein known to be responsible for these activities in Sendai virus. In contrast to cordycepin, the selective inhibition of which, against paramyxoviruses, is considered to be due to a block in the synthesis of the poly(A) sequences of the mRNA, the glucosides did not affect virus RNA or protein synthesis in infected cells. The mechanism of action, further studied by treatment of purified Sendai virions with the most active analogue, p-(sec.-butyl)phenyl-6-chloro-6-deoxy-β-D-glucopyranoside, accompanied by freezing and thawing (which did not affect the biological activities of the virions in the absence of the glucoside), led to drastic decreases of hemolytic and cell fusion activities, as well as infectivity, pointing to reaction of the glucoside with lipid components. This virucidal activity was further supported by using the p-azidophenyl glucoside analogue, also biologically active, and which underwent photoaffinity labelling at site(s) on the virions identified as lipid components in the viral envelopes.

NUCLEOSIDE ANALOGUES

Specific inhibition of viral nucleic acid synthesis has been one of the major goals in antiviral chemotherapy. It is consequently not surprising that so much effort has gone into the synthesis of nucleoside and nucleotide analogues, the more so in that several of these had earlier been shown to be of value in tumour chemotherapy. The first licensed antiviral agent, IUdR, and the first antiviral agent recently licensed for systemic use in man, araA (in biopsy-proven cases of herpes simplex encephalitis), were originally designed as potential antitumour agents. The multitude of analogues available is too extensive to enumerate here, and will be reviewed in part by Dr. J. J. Fox elsewhere in this Symposium. Reference will be made only to recent developments and, in conjunction with the design and use of such compounds in tumour chemotherapy, to those aspects where medicinal chemists are applying synthetic procedures as "rational" as feasible at the moment.

Selected Compounds and their Design

Many of the active nucleoside analogues are relatively specific towards herpes viruses, but there are some with a broader, or even very broad (like ribavirin, see above) spectrum of activity at least *in vitro*. The *in vitro* activities and cellular toxicities (reflected by inhibition of host cell DNA synthesis) of several of the more promising ones, relative to IUdR and araA (which are licensed), are listed in Table 1. It will be noted that the most effective against HSV-1 is

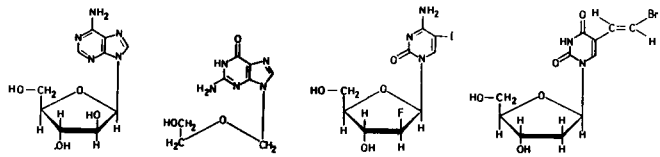


TABLE 1 Inhibitory Effects of Some Nucleoside Analogues (Expressed as ID_{50} in µg/ml) against HSV-1, HSV-2 and Vaccinia Virus Replication in Primary Rabbit Kidney Cell Cultures, and on Host Cell DNA Synthesis

Analogue	HSV-1[+]	HSV-2[+]	Vaccinia	Host Cell DNA Synthesis
AraA	7	5	0.4	7
5-Iodo-2'-deoxyuridine	0.13	0.3	0.3	0.25
5-CF_3-2'-deoxyuridine	0.7	0.7	0.3	0.01
5-Vinyl-2'-deoxyuridine	0.018	0.1	0.4	7
5-Bromovinyl-2'-deoxyuridine	0.008	1	7	20
5-Iodovinyl-2'-deoxyuridine	0.012	2	10	20
5-Ethyl-2'-deoxyuridine	0.5	0.3	1	6
2'-Fluoro-2'-deoxy-5-iodo-araC	0.017	0.05	10	35
Acycloguanosine	0.04	0.04	70	2
5-Iodo-2'-deoxycytidine	0.06	0.3	4	9

[+]Figures are the means for 11 different strains of HSV-1 and 7 different strains of HSV-2. More detailed results for these and other analogues are presented elsewhere (De Clercq and others, 1980).

BVDU (De Clercq and others, 1979), followed closely by the 5-vinyl and 5-iodovinyl derivatives. But acycloguanosine (Fyfe and others, 1978) and FIAC (Lopez and oth-

ers, 1980) are almost equally effective, in this case against both HSV-1 and HSV-2, but much less so against vaccinia. From the last column in the table, which gives the dose required to reduce host cell DNA synthesis by 50%, it will be seen that this is much higher than the required antiviral dose, so that these analogues exhibit a high therapeutic ratio. The situation for araA and IUdR is less favourable. Because of its known mutagenicity, and ability to induce oncogenic viruses following its incorporation into DNA, it is likely that IUdR will in any event shortly be superseded by one of the now numerous promising alternative candidates. Note that 5-ethyl-2'-deoxyuridine, one of the earlier agents synthesized for this purpose (Gauri and Maloney, 1967; Shugar and others, 1967) is still reasonably active against vaccinia; it has been shown to be effective against herpes simplex encephalitis in normal and immunosuppressed mice (Davis and others, 1978). It must be emphasized that the *in vitro* data in the table, although valuable and essential in preliminary screening, will not necessarily reflect the same picture on extrapolation to *in vivo* conditions, and subsequently to clinical trials. The relative activities of the different compounds may even vary appreciably when assayed on a different host cell. One of the reasons for this is intracellular metabolism, which may, for example, lead to inactivation of an amino nucleoside by enzymatic deamination and/or activate it by phosphorylation by viral and/or cellular kinases. Some specific illustrations will now be presented.

Acyclic nucleosides. The finding that 9-(2-hydroxyethoxymethyl)guanine (Acyclovir), an acyclic analogue of guanosine, is a potent and selective inhibitor of HSV-1 and HSV-2 in vitro and *in vivo* (Fyfe and others, 1978), was rapidly followed by the demonstration that erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), a known inhibitor of adenosine deaminase (North and Cohen, 1978), and (S)-(2,3-dihydroxypropyl)adenine, also a deaminase inhibitor (De Clercq and Holy, 1979), exhibit potent antiviral activities. While initially exciting, it should be noted in retrospect that EHNA was known as a deaminase inhibitor 12 years earlier, and that acyclic nucleosides with biological activities, including some isolated from natural sources, e.g. willardine, eritadenine, had been known for some years. Synthetic procedures for such compounds, intended for use as antimetabolites, were reviewed at an antiviral meeting (Horton and others, 1975) three years before reports on the activity of acycloguanosine. A separate review would now be required to describe the literature of the past three years on new synthetic methods alone, including structural and conformational aspects.

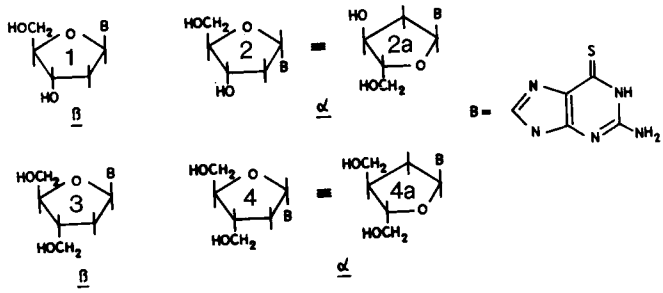
Acyclovir is one of the most potent selective antiherpes agents known. Clinical and *in vivo* trials are actively under way, focussing largely on human tolerance and pharmokinetics, with results pointing to its relative safety. Positive results have been reported for parenteral treatment of 23 immunosuppressed patients with cutaneous and/or systemic herpes simplex or herpes zoster infections (Selby and others, 1979), and positive results in animal studies are being reported almost weekly. Although circumspection should be exercised, at least until pharmacological trials are extended, and that drug-resistant mutants do not pose problems, it is conceivable that introduction of this drug may occur in much less than the 10 years normally required from the lab bench to the clinic.

The mode of action of the compound appears to involve conversion to the 5'-phosphate by HSV virus-coded thymidine kinase, followed by phosphorylation to the 5'-triphosphate and subsequent inhibition of viral DNA synthesis. Lack of activity *vs* vaccinia (see Table 1) is accounted for by the failure of vaccinia-coded thymidine kinase to phosphorylate acyclovir (Fyfe and others, 1978). It is important to note that the substrate properties of acyclovir towards the viral kinase are due to the fact that the acyclic-sugar chain adopts, stereochemically, a non-extended conformations, probably isosteric with natural 2'-deoxynucleosides. Conformations of acyclic nucleosides in relation to biological activity are discussed by Horton and others (1975). Structure-activity relationships have been reported for

a series of acycloadenine analogues (De Clercq and Holy, 1979). Further developments in this active field are to be anticipated.

Pyrimidine nucleosides. The number and variety of antivirally active pyrimidine nucleoside analogues (covered elsewhere in this Symposium by Dr. J. J. Fox) continues to expand at a remarkable rate, and the major problem is now to select, on the basis of pharmacological studies, those most effective. It is particularly gratifying that, in this field at least, a good deal is simultaneously emerging about their mechanism(s) of action, thus providing information essential for development of even more effective drugs. Two of the most promising at the moment, FIAC (Lopez and others, 1980) and BVDU (De Clercq and others, 1979) (see Table 1, above), are initially phosphorylated by HSV-1 thymidine kinase. In the case of BVDU, the 5'-triphosphate has been shown to be a highly selective inhibitor in vitro of the viral polymerase under conditions where cellular polymerases α and β are only minimally affected. The triphosphate inhibited DNA synthesis by competition with the natural substrate, dTTP, the K_i being 0.2 μM as compared to a K_m for dTTP of 0.8 μM. The overall mode of action therefore involves phosphorylation by viral and cellular kinases to the triphosphate, which inhibits the viral polymerase (Allaudeen, De Clercq and others, 1980, in preparation). The low toxicity of the compound is apparently due to the fact that it is a poor substrate for cellular kinase(s), a property common to some other 5-substituted 2'-deoxyuridine analogues. The compound is highly effective against cutaneous herpes infections of athymic nude mice under conditions where the known 5-iodo-2'-deoxyuridine offers only minimal, if any protection. Preliminary clinical trials, against topical infections, are now under way (De Clercq, personal communication).

Anomeric nucleosides. There are now a number of examples of α-anomers of nucleosides as effective, or even more so, as antiviral or antitumour agents, than the corresponding β-anomers (e.g. Peery and LePage, 1969; Shugar, 1974; Bennett and others, 1976; Shannon and Schabel, 1979), and the medicinal chemist would be well advised not to discard the α-anomeric products resulting from some syntheses. While the mode of action of the α-anomers is not immediately obvious, an instructive example, which may form the basis for future investigations, is provided by an elegant study of Acton and others (1979, and references therein).



Amongst thioguanine nucleosides with antitumour properties, the best known are the anomeric pair α-TGdR and β-TGDR, currently undergoing clinical trials. The latter is readily cleaved enzymatically to 6-thioguanine; the former is not and, although less potent than the β-anomer, its lower toxicity permits of its use at higher doses. Its low toxicity is due in part to its unusual selectivity for tumour tissue, where it is phosphorylated and subsequently incorporated into DNA at the trmini of short fragments. It is therefore a substrate for the enzymes which process the β-anomer. On the assumption that the same set of kinases processes both anomers, it was proposed that the commonly assumed gross structural dissimilarity

of the two anomers (1 and 2 in Scheme, above) be re-examined. Structure 2a is formally equivalent to 2 and could then simulate the β-anomer 1, so that phosphorylation would occur on the 3'-OH. Comparison of 2a and 1 suggest the former would better simulate the β-anomer if the 3'-OH were replaced by a 3'-CH_2OH, leading to 4a, which is the 3'-branched analogue 4 of α-TGdR. Both 3 and 4 were synthesized and, in striking accordance with the foregoing reasoning, both equally inhibited growth of W1-L2 human lymphoblastoid cells, were phosphorylated and incorporated to the same extent into the DNA of Mecca lymphosarcoma in mice, and were even more effective than the parent α-TGdR. This system is obviously deserving of further investigation, particularly with regard to enzymological aspects such as the nature and specificities of the kinase(s) responsible for phosphorylation, and the specific phosphatases (3'- and/or 5'-nucleotidases) which hydrolyze the nucleotides of 3 and 4.

Nucleotide analogues. One of the major advantages of nucleoside analogues as antitumour and antiviral agents is the ease with which they are transported across cell membranes. As mentioned above, these nucleosides usually require intracellular phosphorylation by viral and/or cellular kinases to yield the active forms. However, competing intracellular reactions may lead to conversion of a nucleoside to an inactive form prior to its phosphorylation, e.g. araA is frequently deaminated in some cells to the less active, or inactive, araHx. Deamination of araC may be circumvented by use of the 2,2'-anhydro analogue, which is slowly hydrolyzed to the parent araC, so that it is, in fact, a "depot" form of araC. In the case of araA, the carbocyclic analogue (in which the sugar ring oxygen is replaced by carbon) has been found fully resistant to deamination, and to exhibit antiviral activity comparable to that of araA (Vince and Daluge, 1977), a finding which has led to the synthesis of a variety of other carbocyclic nucleoside analogues, too large to enumerate here. Proposals to employ known and effective deaminase inhibitors, widely contemplated, are subject to the reservation that these may interfere with normal metabolic processes of the cell.

There are thus obvious advantages to the use of nucleotide analogues in place of the parent nucleosides. The major difficulty against such an approach is the relative impermeability of cellular membranes to the negatively charged nucleotides. The observation of Cohen and collaborators (see Cohen, 1976, and references therein) that 5'-araAMP is more lethal to mouse fibroblasts than the parent araA, and that this lethal effect is accompanied by transport of a small proportion of the intact nucleotide across the cell membrane, followed by further intracellular phosphorylation and incorporation into cellular DNA, has led to at least some revision of opinion as to the feasability of using nucleotides in place of nucleosides. In support of this, a number of reported, but generally overlooked, examples of transport of organic phosphates across cell membranes have been cited by Cohen. Furthermore, nucleotides are often much more soluble than nucleosides, e.g. one of the disadvantages of araA for systemic antiviral therapy is its low solubility, and there are reported instances of the succesful replacement of araA by araAMP.

There is now a fairly extensive literature on the use of nucleoside monophosphates, 2',3'- and 3',5'- cyclic phosphates and dinucleoside monophosphates as antitumour and antiviral agents. On the assumption that a reduction in negative charge would facilitate transport, a variety of alkyl esters and phosphonates have been synthesized and subjected to biological trials. Although the reported results are not always in agreement with expectations, this field, recently reviewed (Kusmierek and Shugar, 1979), has at the least turned up some analogues which may be profitably pursued further. In addition, permeabilization systems for mammalian cells are now available which should facilitate investigations on the mechanism(s) of action of nucleotide, as compared to nucleoside, analogues. The foregoing should not be confused with the well-known findings of LePage and others (1972) on the therapeutic use of 5'-nucleotides in man, dictated on the premise that nucleotides

do not enter intact cells. The use of nucleotides (of araA and ara-6-mercaptopurine) was actually based on the observation that phosphomonoesterase levels in human kidney are much lower than in the mouse and other small mammals. Administration of the nucleotides to patients led, in accordance with expectations, to sustained blood plasma levels of the nucleosides, as well as better dosage formulations because of the higher solubility of the nucleotides.

PRODUCTS OF NATURAL ORIGIN

Many substances of natural origin have been reported to exhibit antiviral activity (reviewed recently by Swallow, 1978; Becker, 1980) but, unlike the situation in the field of tumour chemotherapy, none (with the possible exception of the interferons) has made its way to the clinic. Some, like aphidicolin (a specific inhibitor of polymerase α), and sinefugin (a potent inhibitor of mRNA cap methylating enzymes) have proven useful as biochemical tools, and sinefugin analogues may prove to have some applications in viral chemotherapy (Borchardt, 1980). Some of the arabinofuranosyl nucleosides, e.g. araU, araT, araA, which are promising or established antiviral agents, are found in natural sources, but they were initially introduced by the synthetic organic chemist. However, present-day large-scale production of araA for clinical use is by fermentation methods (Parke-Davis).

Interferons

The subject of interferons and interferon inducers is too extensive to deal with here. It is, however, difficult to refrain from at least underlining the remarkable advances of the past three years in elucidating the mechanism of action of interferons, as well as the current projects for large-scale production of interferon by conventional methods and by DNA recombination techniques, largely for clinical trials. Numerous claims have been made for effectiveness of interferon in antitumour and antiviral chemotherapy, but in most instances with very impure preparations (see Stewart, 1979). The trials now being planned, or under way, should certainly provide more concrete information. One interesting question is whether interferon produced by recombination techniques, hence non-glycosylated, will exhibit adequate activity in clinical trials.

Interferon inducers. A new and novel series of low-molecular weight interferon inducers, with concomitant antiviral activities, embraces a number of analogues of a pyrimidine, 2-amino-5-bromo-6-methyl-4-pyrimidinol, or simply 5-bromo-6-methylisocytosine. This compound, and several others from amongst 75 with modified 5- and 6- substituents, induced high levels of interferon in cats and mice on oral or parenteral administration. The parent compound was active in both animals and cell cultures, and protected mice against a number of unrelated viruses, but maximum tolerated doses were only 5-fold higher than the minimal effective doses. Subsequent structure-activity studies turned up an additional series with 6-phenyl substituents exhibiting higher activities, and lower toxicities. There was, however, no parallelism between interferon induction and antiviral activity, e.g. the highly antiviral effective 5-iodo-6-phenyl derivative (against Semliki Forest virus and HSV-1) was a poor interferon inducer. Since interferon is known to be effective against RNA viruses (like Semliki Forest) but less effective against DNA viruses (like HSV-1), it appears that some alternate and/or additional mechanism than merely interferon induction must account for antiviral activity (see Stringfellow and others, 1980, and references therein). No tests on primates, or results on mechanism of action, have been reported. It should, however, be noted that the 6-phenyl derivatives are formally analogous to some potent polymerase inhibitors, 6-arylpyrimidines (Brown and Wright, 1977; Wright and others, 1980), although this may be coincidental. Furthermore a number of substituted pyrimidines have been reported as antivirals, a good example being ethyl-2-methylthio-4-methyl-5-pyrimidine, a

selective inhibitor of polio virus, by blocking of cell-mediated eclipse (Lonberg-Holm and others, 1972), but not tested for interferon induction.

VIRAL ENZYME INHIBITORS

Inhibition of a key enzyme in the virus replicative cycle is one obvious goal in chemotherapy of viral diseases, but this was clearly recognized only 10 years ago with the discovery of the reverse transcriptases of retraviruses, which provided the necessary impetus for investigation of the properties of viral-associated and induced enzymes, extensively reviewed by Kit (1979). A brief account of viral nucleolytic enzymes has appeared (Sierakowska and Shugar, 1977). Examples of inhibitors that discriminate against one member of a given class of enzymes have already been cited above, e.g. PAA and PFA, which selectively inhibit HSV DNA polymerases relative to the cellular enzymes; acycloguanosine and BVDU, which are initially phosphorylated by HSV, but not cellular, kinases, and the triphosphates of which specifically inhibit the HSV, and to a much lesser extent cellular, polymerases. But probably the best illustration of the ability to design specific polymerase inhibitors is provided by the 6-(arylhydrazino)pyrimidines, originally exploited for specific inhibition of bacterial polymerases (see review by Brown and Wright, 1977) and presently being extended to the field of mammalian polymerases through 6-anilinouracils (Wright and others, 1980). These classes of inhibitors have been singled out for mention here because their mechanism of action has been extensively investigated and utilized for the design of new inhibitors. Additional such examples include inhibitors of thymidylate synthetase (reviewed by Santi, 1980), although these will probably be more useful in tumour chemotherapy; and agents which inhibit macromolecular methylation, the S-adenosyl-L-methionine-dependent methyltransferases (reviewed by Borchardt, 1980). Bearing in mind the key role of reverse transcriptase in the replicative cycle of RNA tumour viruses, it is not surprising that so much effort is being devoted to the search for specific inhibitors of this enzyme (e.g. Chandra and others, 1977).

ACKNOWLEDGEMENTS

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Chemical Control of Oncogenic Events by RNA Tumor Viruses

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ABSTRACT

The present state of our knowledge on the replicative cycle of oncornaviruses (RNA Tumor Viruses: Retroviruses), and the molecular events involved in cell transformation by such viruses, suggests several points of attack in blocking the oncornavirus-induced transformation or the expression of integrated viral information (Oncogene) in the genome of the host cell.

The life cycle of the oncornaviruses involves the following sequential events: 1) Adsorption and penetration of the virus into the host cell; 2) Release of viral components followed by the synthesis of proviral DNA; Integration of the proviral DNA segment into the host genome; 4) Transcription and processing of virus-related RNA; 5) Translation of viral proteins; 6) Assembly of proteins and RNA; and 7) The envelopment and release (budding) of the virus particles.

Adsorption is mainly dependent on the recognition of the host cell surface receptors by the viral envelope proteins. Our present knowledge about the factors which govern the relationship between the viral envelope proteins and the cell surface receptors is still in its infancy; hence a molecular strategy to block this process is difficult. However, this process is amenable to specific immunological control.

The next step in the life cycle, formation of proviral DNA, is unique to this class of viruses and is therefore most amenable to chemical control. One of the major concerns of our laboratory is the development of chemical inhibitors which block the synthesis of proviral DNA. These studies will be described here in detail.

1. INTRODUCTION

Nucleic acids, or nucleic acid polymerizing enzymes, have served as extremely useful targets in the development of chemotherapeutic agents to combat infections and malignant diseases. In some instances, such as antiviral drugs, this approach has provided virtually the only means to design effective chemotherapeutic agents. As a matter of fact, it is one of the most significant contribution of molecular biology, as a basic discipline, to prsent-day medical sciences.

The strategical role of nucleic acids, or nucleic acid polymerases as targets, involves two distinct features. The investigational drug must have the capacity to recognize distinct bases or base-pair sequences, either by direct interaction between functional groupings on the base-pairs and the drug molecule, or indirectly via recognition of the conformational peculiarity of the nucleic acid molecule. The other alternative is the specific affinity of the drug for one or the other nucleic acid polymerizing enzymes. From the strategic standpoint this is a more specific approach, since a number of enzymes are involved in the polymerization of nucleic acids.

The fact that RNA tumor viruses are endowed with both the novel features, i.e. an RNA of a specific size (70S) and a DNA-polymerase peculiar to such viruses only, offers a very meaningful approach to develop specific antiviral compounds.

The very fact that the enzyme catalyzing RNA-templated DNA synthesis is present in all oncornaviruses, suggests its role in the neoplastic transformation by such viruses. The DNA copy (Provirus) of the viral genome (RNA) synthesized by this enzyme is incorporated into the host cell DNA where it carries information for viral replication and information for transformation of the normal cell to a neoplastic cell (Fig. 1)

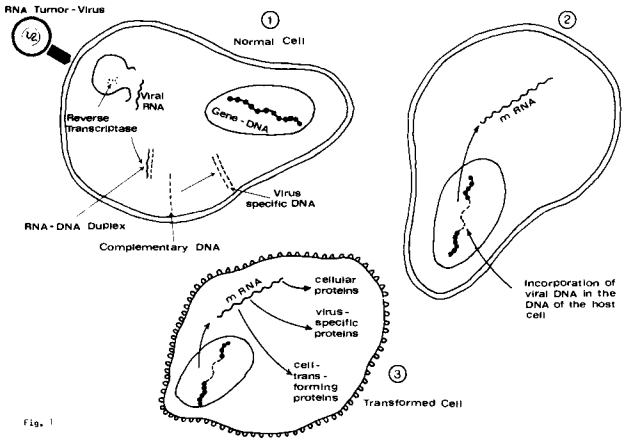


Fig. 1. Schematic presentation of oncogensis by RNA tumor viruses.

2. APPROACHES TO INHIBITING ONCOGENESIS BY RNA TUMOR VIRUSES

There are several approaches to inhibiting the molecular processes leading to a manifestation of oncogenic information. Of these, two approaches are of interest from the experimental standpoint: (1) inhibition of the expression of proviral DNA, and (2) Inhibition of proviral DNA synthesis.

2.1. Inhibition of the Expression of Proviral DNA

The integrated viral-specific DNA can be transcribed to produce viral RNA which serves as messenger to code for the virus-specific structural proteins, as well as proteins required for the transformation process. The molecular reactions which lead to the formation of viral RNA and its subsequent translation are shown in Fig. 2

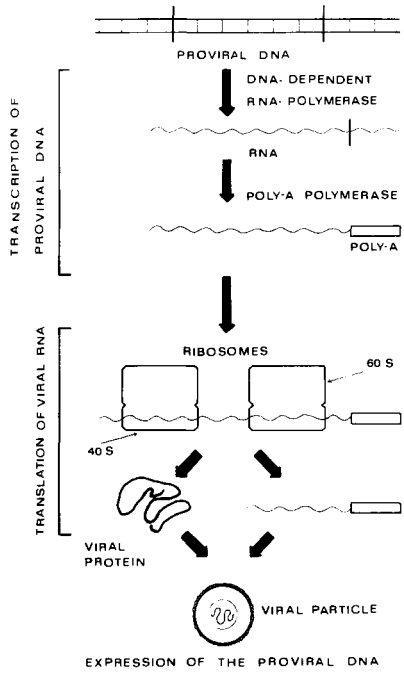


Fig. 2. Process of proviral DNA expression.

Wu et al. (1972) have shown that cordycepin (3′-deoxyadenosine) blocks the induction of virus production by 5-iodo-2′-deoxyuridine ; other adenine containing nucleosides did not block this induction. It was suggested (Wu et al., 1972) that cordycepin acts by inhibiting the formation of polyadenylic acid (poly A) by blocking chain extension due to the absence of 3′-OH groups. Since poly A sequences are a characteristic feature of all RNA tumor virus genes, such inhibitors are of future interest. However, the selectivity in blocking the proviral DNA expression may be argued, since poly A sequences are known to occur in normal cellular messenger RNA (Darnell, 1968). It

has been suggested that poly A stretches are required for transport of the messenger RNA from nucleus to cytoplasm (Darnell, 1968). If the same poly A-polymerase synthesizes the poly A sequences for the viral RNA and for cellular messenger RNA, it is questionable as to whether this adenosine analogue can slectively block the expression of proviral DNA.

2.2. Inhibition of Proviral DNA Formation

It may be possible to inhibit proviral DNA formation at several stages (see Fig. 1): (a) blocking the penetration of the membrane by the virus; (b) blocking the release of viral components inside the host cell; (c) inhibiting the RNA-dependent DNA polymerase activity; and (d) damaging the genomic RNA of the virus. The last two modes of inhibition are of special interest since the reverse transcriptase and the 70s RNA are novel features of all oncornaviruses.

Although it has been only recently recognized that the process of reverse transcription offers a unique target for drug design, the number of compounds reported to inhibit this process has exceeded expectations. It is indeed very encouraging that this approach has been endowed with so much experimentation in a number of laboratories; however, the critical analysis of the en block progress leaves a big gap.

The in vitro assay systems employed for reverse transcriptase (RT) determination reveal a variety of substrates, template-primers and interacting compounds which can modulate the catalytic rate of of DNA synthesis. Some examples of this type of modulation are: the detergent effect on the activity of rifamycins (Thompson et al.,1972), influence of divalent cations (magnesium or manganese ions) on the rate of DNA synthesis with different substrates and the role of chelating agents or cation binders in buffers (see Temin and Baltimore, 1972), and interaction of thiols with some potential inhibitors or their direct influence on the measured DNA synthesis (Levinson et al., 1973, 1977). Thus slight variations in assay conditions may lead to wrong interpretations with respect to the specificity of a particular inhibitor in the viral DNA-polymerase system.

The second problem is the interpretation of enzymatic data with respect to the antiviral activity of these inhibitors (Chandra et al., 1977a, 1977b). This is particular the case with such compounds which exert their inhibitory action by complexing with one or more synthetic templates. Such an effect may be due to lack of specificity of such inhibitors for the viral enzyme. Drugs exhibiting cytotoxic activities to the extent of causing a delayed death, or those which intervene in the replicative cycle of the host cell may give erroneous information about the specific antiviral activity of the compound. Thus, antiviral studies in vitro should be carried out under conditions and at inhibitor concentration which have little or no effect on the replicative cycle of the cell. Molecular manipulations of parent compounds have proved to be very useful in several instances to develop inhibitors of viral DNA polymerases which exhibit a low cytotoxicity, and at the same time high antiviral potential. This is evidenced by our studies on distamycin derivatives(Chandra et al., 1972a, 1972b, 1972c, 1972d, 1975a, 1977a, 1977b; Chandra 1974a), daunomycin derivatives (Chandra et al., 1972b, 1972e, 1975a, 1977a, 1977b; Chandra, 1974a, 1974b, 1975) and tilorone congeners (Chandra et al., 1972f, 1972g, 1972h, 1974a; Chandra, 1977a, 1977b; Chandra,Woltersdorf and

Wright, 1979).

Further efforts to develop compounds which inhibit viral DNA polymerases by interacting with templates may lead to the discovery of useful compounds exhibiting a higher therapeutic index, i.e. low cytotoxicity and high antiviral potential. However, this approach will not lead to the development of a specific inhibitor of the viral enzyme, unless one finds a compound which binds specifically to the genomic viral RNA. Though 70s RNA is a novel feature of oncornaviruses from the chemical standpoint it does not appear to offer any uniqueness to distinguish it from cellular nucleic acids. Thus, the strategical approach of developing an inhibitor of this type is, at the present state of our knowledge, unthinkable.

The second molecular approach, which has proved to be more useful and relatively specific in developing such inhibitors is to design compounds that bind to the viral enzyme. In searching for this type of inhibitor, the enzymes chosen for comparison are important. Many studies have been done with either avian or mammalian viral reverse transcriptases, since the two enzymes have some different characteristics. A number of studies claim specificity (or selectivity) of a compound by comparing an inhibitory response to a bacterial DNA polymerase with the viral reverse transcriptase. Such a comparison has no relevance to the selectivity of the compound. The most important approach to demonstrate the selectivity of a compound is to compare its inhibitory effects against various eucaryotic cellular DNA polymerases such as α- , β-, and γ- DNA polymerases.

3. CLASSIFICATION OF INHIBITORS

Compounds that inhibit viral DNA polymerase can be classified according to their mode of action (Smith and Gallo, 1974), or can be subdivided into various groups on the basis of their chemical structure (Chandra et al.1975a, 1977a, 1977b).

Depending upon their mode of action the inhibitors of viral DNA polymerase can be divided into six classes (for references to various compounds, see Chandra et al., 1977a, 1977b, 1980):

(1) Enzyme-binding compounds: Ansamacrolides (Rifamycins, Streptovaricins), Ca-elenolate, Alkaloids , Pyran copolymer.

(2) Substrate analogues: Ara-CTP, 2′-3′-Dideoxythymidine triphosphate (ddTTP)

(3) Template-Primer analogues: Oligothymidylate derivatives, polyribonucleotides, analogues of polyadenylic acid, partially thiolated polycytidylic acid, Vinyl analogues of polynucleotides.

(4) Template binding compounds: Actinomycin D, Chromomycin and Olivomycin, Daunomycin and derivatives, Distamycin and derivatives, Tilorone and congeners, Ethidium bromide, Proflavin, Cinerubin, Fagaronine, Ionophorous polymers, Diamidine Phenylindole.

(5) Divalent cation-binding agents: O-Phenanthroline, Thiosemicarbazones

(6) Miscellaneous: Streptonigrin, 2-Oxopropanol, Silicotungstate, 5-Tungsto-2-animoniate.

Due to limited space, we will report only our recent studies with some substrate analogs of nucleic acid bases, and some modified polynucleotides acting as anti-templates. For other compounds, the reader is referred to the reviews which contain most of the studies carried out with other compounds (Chandra et al., 1977b, 1980).

4. EFFECT OF 5-ALKYL-2´-DEOXYURIDINE 5´- TRIPHOSPHATES ON THE DNA-POLYMERIZING ENZYME ACTIVITIES.

The use of chemically modified deoxynucleoside triphosphates as analogs of nucleic acid bases, is a well-known strategy to block DNA synthesis catalyzed by the DNA polymerase reaction, or synthesize DNA species with altered functional activity. However, in view of their importance as antiviral compounds, only such substrate analogs are of value which are specifically utilized by the viral induced enzymes. Using this approach, Cheng et al. (1976) described significant slectivity of 5-alkyl-substituted deoxyuridine analogs against herpes-virus-induced enzymes; 5-Ethyl-Deoxyuridine (Et.-dU) and 5-Propyl-Deoxyuridine (Pr.-dU) are known to possess antiherpetic activities(Gauri, 1979). In view of this, we were interested to look for the substrate-replacement activity of 5-Ethyl- and 5-Propyl-analogs of deoxyuridine triphosphates (Fig. 3) in the DNA-polymerase reaction catalyzed by the cellular DNA polymerases (polymerase- α, -β, and -γ), and by the virus-induced reverse transcriptase (RT).

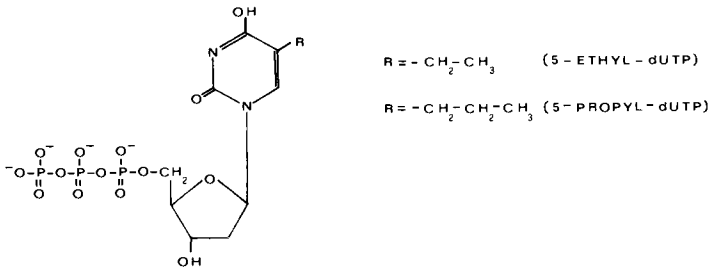


Fig. 3. Chemical Structures of 5-Alkyl-Deoxyuridine Tri-Phosphates.

The studies reported herein were carried out using DNA-polymerase-α from calf thymus, DNA-polymerase-β from the Rhabdomyosarcoma tissue of a child, DNA-polymerase- γ from the human ovarian tumor tissue, and the viral reverse transcriptase (RT) from RLV-infected spleen. All the enzymes were purified by column chromatographic procedures followed by isoelectric focusing (Chandra et al., 1980a, 1980b; Chandra and Steel, 1977). The purity of these enzymes was characterizedby SDS-gel electrophoresis and using specific primer-templates in their assay systems.

As follows from Fig. 4, the 5-Ethyl-derivative (ET-dUTP) inhibits the reverse transcriptase (RT) reaction catalyzed by $(rA)_n.(dT)_{12}$ significantly; whereas, under similar experimetal conditions 5-Propyl-derivative (Pr.-dUTP) fails to inhibit the RT-reaction.

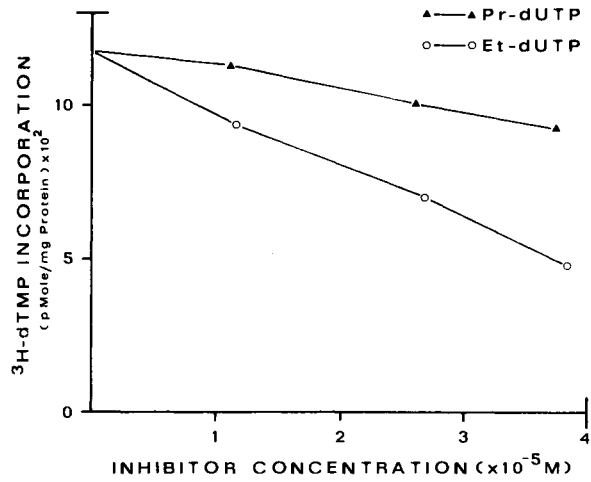


Fig. 4. Inhibition of $(rA)_n.(dT)_{12}$-catalyzed reverse transcriptase activity from RLV-infected spleen by 5-alkyl-deoxyuridine triphosphates.

The kinetics of RT-reaction plotted according to Lineweaver-Burk equation (Fig. 5) shows that the inhibition by ET-dUTP is of competitive nature (K_m = 0.4x10^{-10}; Ki = 0.166x10^{-15}M).

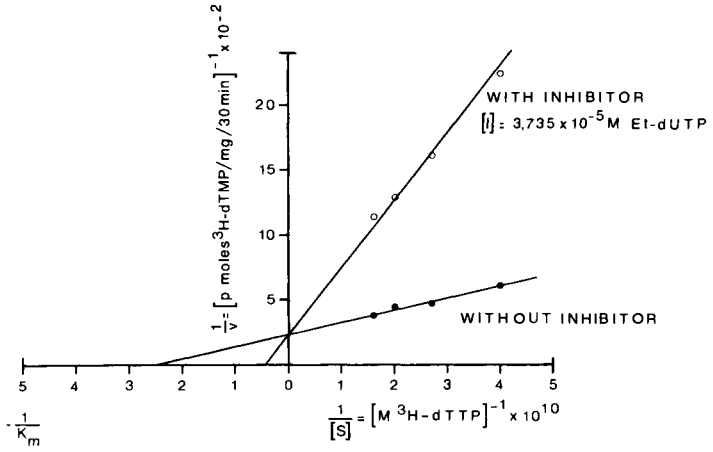


Fig. 5. Lineweaver-Burk plot of the kinetic data of RT-reaction inhibited by ET-dUTP.

The inhibition of cellular DNA polymerase -α, -β and -γ are shown in Figures 6-9. As follows from these results, the DNA-polymerase -α is least sensitive to inhibition by both the analogs; only ET-dUTP

shows some inhibition whereas, the Pr.-dUTP derivative is almost ineffective at the concentrations used. The γ-polymerase reaction is sensitive to both the analogs; however, ET-dUTP is slightly more effective than Pr.-dUTP - at least,at lower concentrations.The β-polymerase reaction is most sensitive to both the analogs, but also in this reaction, the ethyl derivative is more effective than Pr.-dUTP.

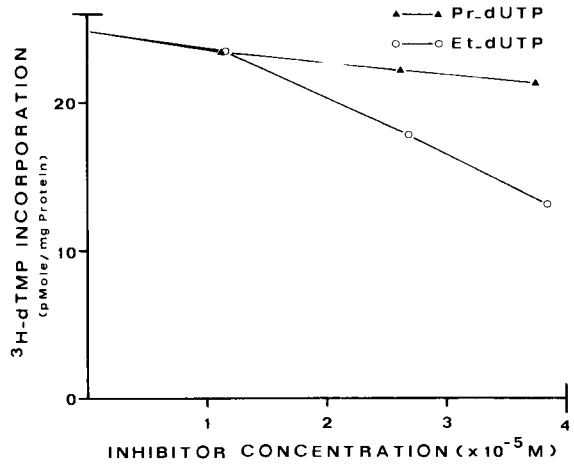


Fig.6 . Inhibition of DNA-Polymerase-α from calf thymus by 5-alkyl-deoxyuridine triphosphates.

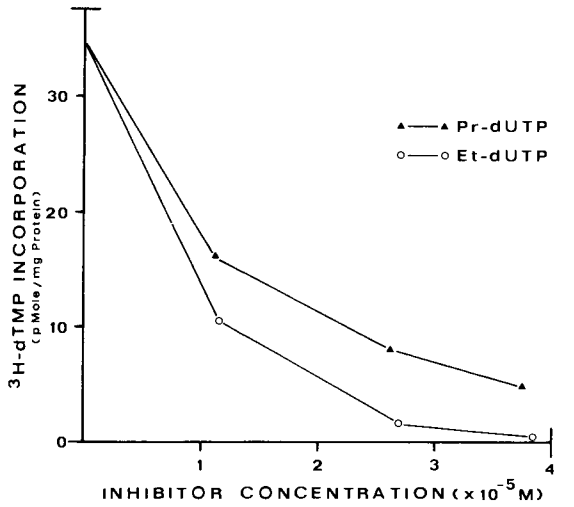


Fig.7 . Inhibition of DNA-Polymerase-β from human Rhabdomyosarcoma tissue by 5-alkyl-deoxyuridine triphosphates.

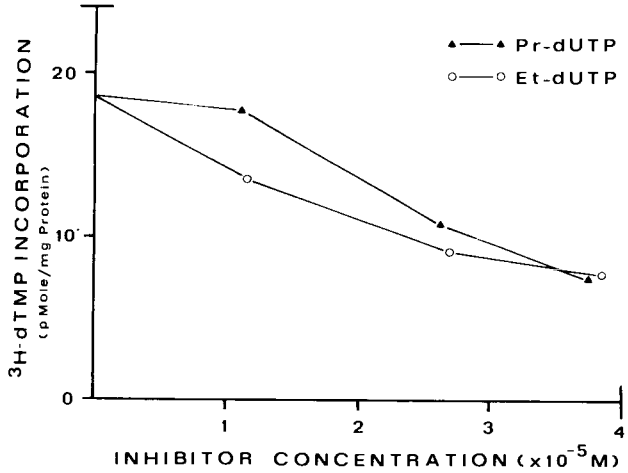


Fig. 8. Inhibition of $(rA)_n.(dT)_{12}$-catalyzed DNA-Polymerase-γ activity from human ovarian tumor tissue by 5-alkyl-deoxyuridine triphosphates.

Since both the analogs showed a high inhibitory activity against the DNA-polymerase-β reaction, we studied the kinetic of this reaction. The results plotted according to Lineweaver-Burk showed an unexpected difference in the mode of inhibitions by the two analogs (Fig. 9). Whereas, ET-dUTP inhibits the reaction competitively, the Pr.-dUTP shows a non-competitive inhibition of the ß-polymerase reaction.

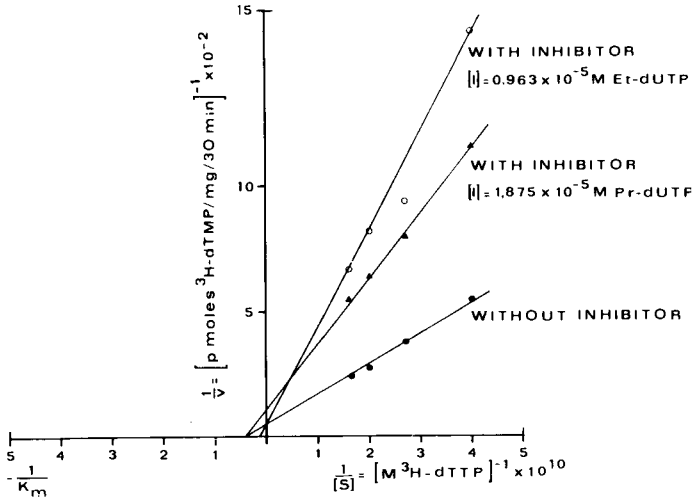


Fig. 9. Lineweaver-Burk Plot of the kinetic data of Polymerase-ß inhibition by 5-alkyl-deoxyuridine triphos.

Summarizing the studies on 5-alkyl-substituted deoxyuridine triphosphates, one can say that the α-polymerase reaction is least sensitive to these analogs; whereas, the ß-polymerase reaction is most sensitive to the action of these compounds. It is interesting that the mode of inhibition of the polymerase-ß reaction by these two analogs is different. The viral induced RT activity is moderately sensitive to ET-dUTP, but not towards Pr.-dUTP. Thus, both the compounds do not satisfy the criteria of selectivity as inhibitors of the virus induced enzyme. Their potentiality as antiherpetic drug may lie in their selective activity towards the thymidine kinases of normal and virus-infected cells, as claimed by Cheng et al. (1979).

In a recent study, Sagi et al. (1980) have reported the effect of a series of 5-alkyl-substituted deoxyuridine triphosphates on the DNA-polymerase-α and -β activities from calf thymus. Similar to our findings, they also observed that DNA-polymerase-α is less sensitive to these analogs. However, in contrast to our findings, they reported that Pr.-dUTP analog is more effective than ET-dUTP. A direct comparison is not possible since these authors (Sagi et al., 1980) did not measure the Ki of these inhibitors in their enzymatic reaction. Recent studies from our laboratory have shown that terminal deoxynucleotidyltransferase (TdT) activity,purified from human thymus,is strongly inhibited by both the analogs of deoxyuridine triphosphate. These studies are still in progress to elucidate the mode of TdT inhibition by the two compounds.

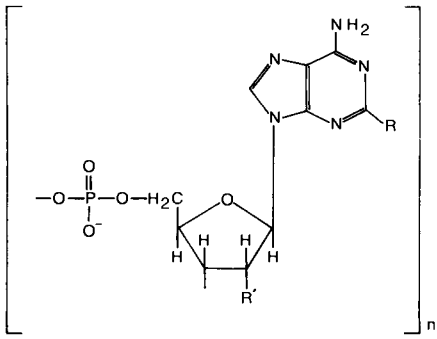
5. ANTITEMPLATE APPROACH TO DEVELOP SELECTIVE INHIBITORS OF DNA SYNTHESIS

Single-stranded polyribonucleotides are known to act as efficient templates for the viral DNA-polymerases in the presence of a complimentary oligo-deoxyribonucleotide primer. Chemical modification of such templates would be expected to alter the anteraction between the template and the viral enzyme (for review, see Chandra et al., 1977b, 1980). This appears to be a very useful approach for designing inhibitors of viral DNA polymerases (Chandra,1974a, 1974c; Chandra et al., 1972 i, 1974c, 1974d, 1975, 1975a, 1975b, 1977b, 1980) which might application in the chemotherapy of cancer (Chandra,Kornhuber & Ebener, 1979).

5.1. Polyadenylic Acid Analogs

The rationale for developing polyadenylic acid (Poly A) analogs is based on the fact, that all oncornaviral DNA polymerases have the property to transcribe poly A efficiently. The transcription of poly-A requires an oligodeoxythymidylic acid primer. Most of these modifications were achieved by substitutions at position 2 of the purine ring, or at 2´-position of the ribose molecule (Arya et al.,1974, 1975; De Clercq et al., 1979). Depending upon the structural modifications, these analogs exhibited a wide spectrum of inhibition of the viral DNA polymerase. However, none of these studies report a comparative analysis of these analogs in other eucaryotic DNA polymerases. As mentioned under Introduction, the comparative analysis of an inhibitor in different polymerase systems is important to evaluate the selectivity of an inhibitor. In view of this, we have made a comparative analysis of three such polyadenylic acid analogs (Fig. 10) in various DNA polymerase systems to evaluate their selectivity as antiviral compounds. These analogs are: poly (2-methylthioadenylic acid: $(ms^2 A)_n$; poly (2-ethylthioadenylic acid): $(es^2 A)_n$; and poly -

(2′-fluoro-2′-deoxy-adenylic acid): $(dAfl)_n$. The activity of these compounds on reverse transcriptase activity of AMV (Boehringer Mannheim, Germany) has been recently reported by De Clercq et al.(1979).



R = −S−CH_3, R′ = −OH: poly(2-methylthioadenylic acid), $(ms^2A)_n$

R = −S−CH_2CH_3, R′ = −OH: poly (2-ethylthioadenylic acid), $(es^2A)_n$

R = −H, R′ = −F: poly (2′-fluoro-2′-deoxyadenylic acid), $(dAfl)_n$

Fig. 10. Chemical structures of polyadenylic acid analogs.

The effect of these three poly A analogs on $(rA)_n.(dT)_{12}$-primed DNA polymerase activity of RLV is shown in Fig. 11. The purified enzyme showed a single peak of activity in the isoelectric focusing column at pH 5.6. As follows from results, $(ms^2 A)_n$ shows a maximum inhibition of the enzyme activity followed by $(es^2 A)_n$. On the contrary, $(dAfl)_n$ showed a concentration dependent stimulation of the RT reaction. However, in the presence of $(rC)_n.(dG)_{12}$ as template-primer, the RT reaction was significantly inhibited by $(dAfl)_n$. This biphasic effect effect is understandable since $(dAfl)_n$ acts in this situation as antitemplate; whereas, this analog does not compete with $(rA)_n.(dT)_{12}$ as antitemplate or, it acts by itself as template alike poly A.

A very similar spectrum of inhibition was observed in the DNA-polymerase-γ reaction (Fig. 13), catalyzed by $(rA)_n.(dT)_{12}$. Both the 2-substituted derivatives inhibited the γ-polymerase reaction strongly, whereby the $(ms^2 A)_n$ analog was a better inhibitor. The 2′-substituted derivative, $(dAfl)_n$, showed a concentration dependent stimulation of the DNA polymerase-γ reaction.

The main conclusion from these experiments is that, $(es^2 A)_n$ and $(ms^2 A)_n$ inhibit the $(rA)_n.(dT)_{12}$-catalyzed reactions (RT and the DNA polymerase- γ), whereas the $(dAfl)_n$ derivative stimulates the $(rA)_n.(dT)_{12}$-dependent reaction. It was therefore of interest to look for their relative activity in the DNA polymerase-B reaction, catalyzed by $(dA).(dT)_{12}$. As follows from Fig. 14, the polymerase-B reaction is inhibited by all the three analogs. The fact that $(dAfl)_n$ inhibits the $(dA)_n.(dT)_{12}$-catalyzed reaction indicates that $(dAfl)_n$ may have a secondary structure similar to $(rA)_n$, than to $(dA)_n$. On

the other hand, we have recently found that the oligo dA-catalyzed activity of TdT from human thymus is not inhibited by $(dAfl)_n$.Thus, $(dAfl)_n$ inhibition may serve as a good indicator for the detection of DNA polymerase-ß in cells and tissues.

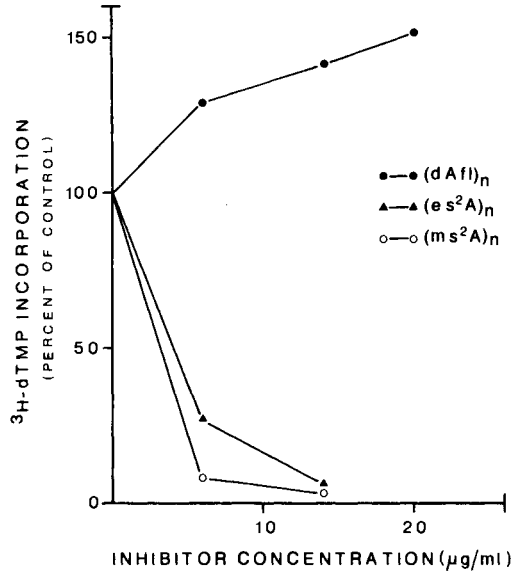


Fig. 11. Effect of 2- and 2´-substituted poly A analogs on the $(rA)_n.(dT)_{12}$-dependent activity of reverse transcriptase from RLV-infected spleen.

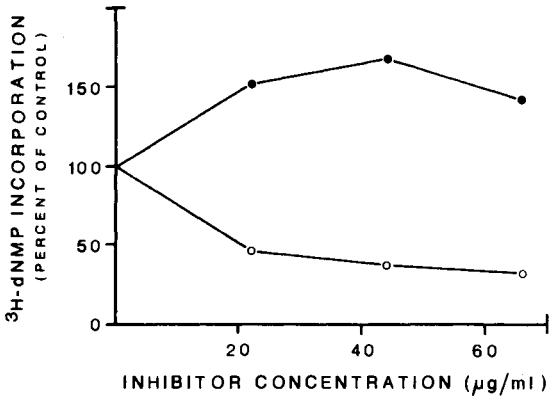


Fig. 12. Effect of poly (2´-fluoro-2´-deoxy-adenylic acid) :(dAfl): on the exogenous activity of reverse transcriptase (RLV), catalyzed by $(rA)_n.(dT)_{12}$ ●——●; and $(rC)_n.(dG)_{12}$ O——O.

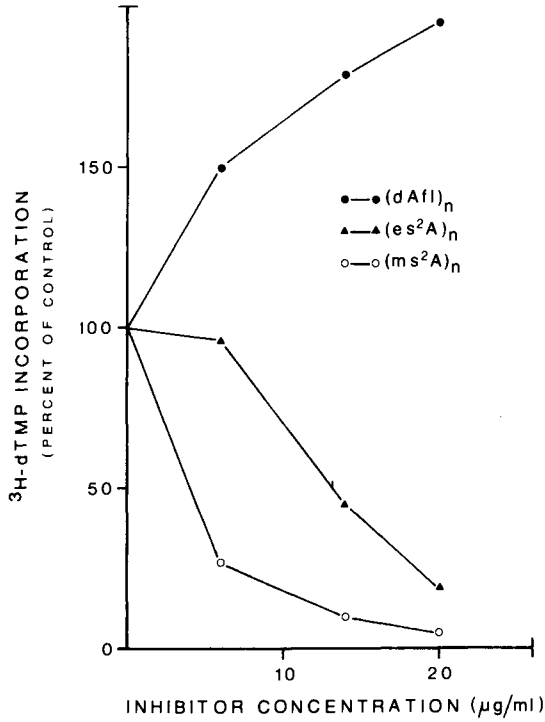


Fig. 13. Effect of 2- and 2′-substituted poly A analogs on the $(rA)_n.(dT)_{12}$-catalyzed activity of DNA polymerase- γ from human ovarial tumor tissue.

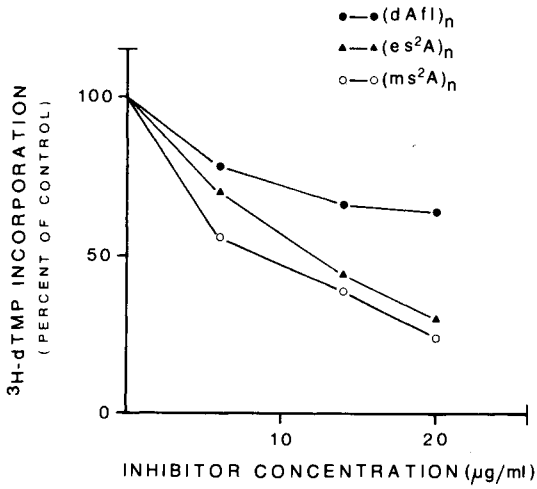


Fig. 14. Inhibition of $(dA)_n.(dT)_{12}$-catalyzed polymerase-ß reaction by poly A analogs.

5.2. Inhibition of Oncornaviral DNA Polymerase by 5-mercapto-Poly C .

Our efforts to develop compounds that inhibit viral DNA polymerase by interacting directly to the enzyme led to the discovery of a polycytidylic acid analog, containing 5-mercapto substituted cytosine bases (Fig. 15), a partially thiolated polycytidylic acid. This compound, abbreviated as MPC, was found to inhibit the onrcornaviral DNA polymerase in a very specific manner (Chandra et al., 1972i, 1974 c, 1974d, 1975, 1975a, 1975b, 1977b; Chandra, 1974a, 1974c 1979).

The inhibition of DNA polymerases from RNA tumor vriuses by MPC was studied using different MPC samples with varying degree of thiolation. The inhibitory actvities were directly proportional to the degree of thiolation; a maximum inhibition was observed with samples containing 15-17% of the thiolated bases.

The mode of action of MPC on the inhibition of viral DNA synthesis was investigated by the product analysis of the DNA polymerase reaction in the absence or in the presence of MPC, as described elsewhere (Chandra etal., 1975b). Analysis of the endogenous products of the detergent disrupted virions on hydroxylapatit column exhibits 3 DNA species: single stranded DNA (first peak), RNA-DNA hybrid (2nd peak) and the double stranded DNA (third peak). As follows from Fig. 16, in the presence of MPC (open circles) there is an overall inhibition of ^{3}H-TMP incorporation, indicating that the formation of all 3 species is blocked. This is to be expected since the inhibitor binds to the enzyme. This has been confirmed by our ultracentrifugation experiments in which the binding of ^{35}S-labeled MPC to a purified FLV-DNA polymerase was investigated (Fig. 17).

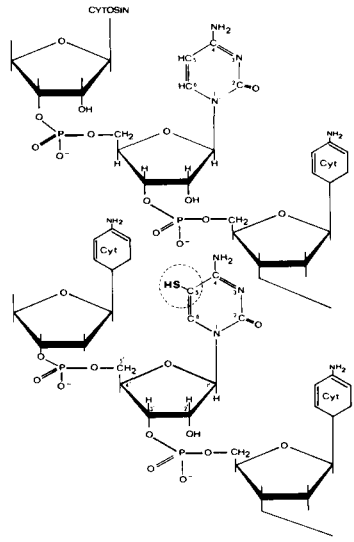


Fig.15. Schematic presentation of chemical structure of 5-mercapto-poly C(lower chain

In order to determine the selectivity of MPC action, cellular DNA polymerases α, β and γ were purified from the same tissue, and the effect of MPC on their enzymatic activies was studied under optimal conditions. The effect of MPC on the cellular DNA polymerases of normal mouse spleen, and spleen of mice infected with FLV is shown in Fig. 18. As follows from these results, there is a slight, or no inhibition of the cellular polymerases from either source; however, a strong inhibition of $(rA)_n.(dT)$- and $(rC)_n.(dG)$-catalyzed activities was observed (Fig 18, curves 6 & 7).The kinetic analysis of these results by the Lineweaver-Burk equation showed that MPC-inhibition is of a non-competitive nature.

The studies on the selectivity of MPC action were substantiated using the cellular DNA polymerases and a reverse transcriptase from human spleen. We have recently discovered a reverse transcriptase in the spleen of a patient with myelofibrosis(see Chandra, 1980a). The human reverse transcriptase activity was far more sensitive to MPC,

than the corresponding cellular DNA polymerases.

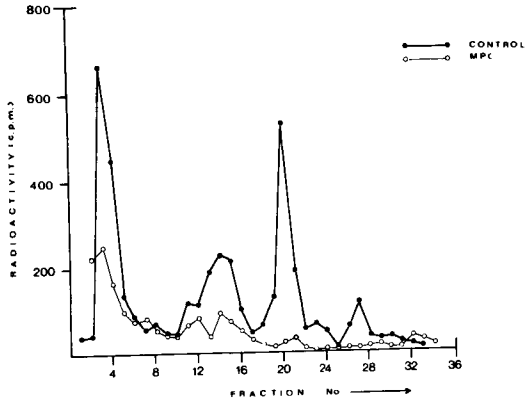


Fig. 16. Analysis of the DNA species synthesized by FLV-DNA polymerase by elution from hydroxyl-apatit column.Details are described in the text.MPC concentration= 20 µg/ml.

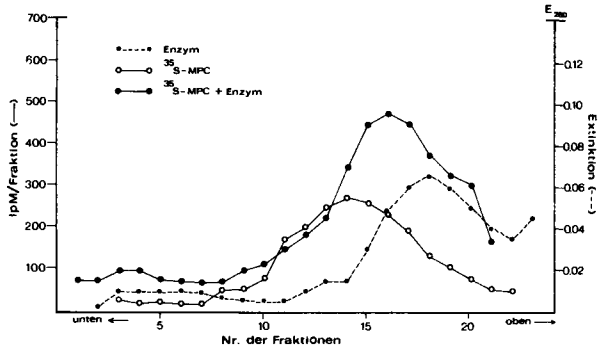


Fig. 17. Binding of ^{35}S-labeled MPC to a purified FLV-DNA polymerase.The MPC specimen contained 10.1% of thiolated cytosine bases (sp.Act. 141 c.p.m. per µg MPC). Other details are described elsewhere (Chandra, 1979).

5.2.2 Effects of MPC on oncogenesis by RNA tumor viruses: To measure the effect of MPC (SH= 8.6%) on the production of splenomegaly by Friend leukemia virus (FLV), we have carried out a series of biological studies (see Chandra et al, 1980); the important ones are documented in Table 1. The animals were divided into four groups of five each(donors): 1. Group 1 was injected with a viral suspension (citrate plasma from FLV infected animals, dose LD_{90}) preincubated with Tris/HCl buffer, pH 7.6 for 30 min. at 37 C^{o}. 2. Group 2 was injected with the viral suspension, as in 1, but preincubated with MPC (200 µg per 0.2 ml suspension) at 37 ^{o}C for 30 min. These animals received in addition, on day 5 and day 9 (post infection) 50 µg of MPC, injected intraperitoneally; 3. Group 3 was treated similar to

group 1, except that the viral suspensions were preincubated for 2 hrs.; 4. Group 4 was treated in a similar manner as group 2, except that the viral suspensions were preincubated for 2 hrs at 37 °C. On the 10th day, animals were sacrificed and spleen extracts were prepared, as described earlier (Chandra et al., 1975). The spleen extract from each mouse was then analyzed individually, with respect to their leukemogenic potentiality. Each "donor" spleen specimen was reinjected to a different "receipient" mouse (20 in total), and the leukemogenesis was followed, as shown in Table 1.

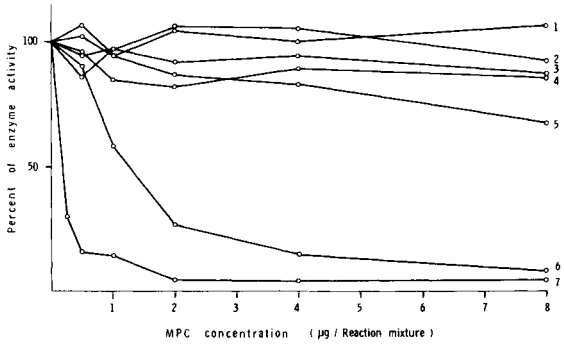


Fig. 18. Effect of MPC on the activity of cellular DNA polymerases and RT from normal and FLV-infected mouse spleen. Cellular DNA polymerases from normal spleen are depicted by curve 1(α), 2(β) and 4(γ); cellular DNA polymerases from FLV-infected spleen are depicted by curve 3(α) and 5 (β); Reverse Transcriptase activities catalyzed by $(rA)_n.(dT)_{12}$ (curve 7) and $(rC)_n.(dG)_{12}$ (curve 6).

T A B L E 1

ASSAY FOR LEUKEMOGENIC POTENTIAL OF SPLEEN EXTRACTS FROM FLV-INFECTED MICE AFTER THEIR IN-VITRO/VIVO TREATMENT WITH MPC.

TREATMENT OF DONOR MICE	LEUKEMOGENESIS IN RECEPIENT MICE AFTER INFECTION WITH SPLEEN EXTRACT[1]		
	NO. OF POSITIVE / TOTAL NO. OF MICE	MEAN SURVIVAL TIME (DAYS)	MEAN SPLEEN WEIGHT(g)
Virus Suspension[2] (0.2 ml) + Tris buffer (37°C, 30 min)	5/5	47	2. 41
Virus Suspension + 200 µg of MPC (37 °C, 30 min.) + 50 µg MPC, i.p. (day 5 & 9)	2/5	123[3]	1. 05 (1,78, 2.10, 0.52,0.41,0.44)
Virus Suspension + Tris buffer (37 °C, 2hr)	5/5	52.2	1. 80
Virus Suspension + 200 µg of MPC (37 °C, 2 hr) + 50 µg MPC, i.p. (day 5 & 9)	1/5	110 4 (123)[3] 1 (97)	0. 38 (0.74,0.29,0.34, 0.22, 0.31)

a Cell-free spleen extracts were prepared as described in Text.
b Citrate plasma from FLV-infected mice was used (LD_{90})
c The experiment was terminated on day 123 and all animals were sacrificed on this day. Therefore, the term mean survival period does not apply to these animals.

All animals in groups 1 and 3 developed splenomegaly and died between 40-60 days; whereas, in the MPC treated groups, of the 10 animals only 3 showed signs of splenomegaly. In group 2, 2 animals had splenomegaly but, in spite of that, all animals survived till the 123rd day, at which time our experiments were terminated. Similarly, in the last group 4 animals survived till the 123rd day; one animal died on the 97th day. The spleen weights, shown in the last column, also exhibit large differences between the MPC-treated group, and the control group. In another study we have analyzed the effect of MPC on normal mice of the same strain.We failed to observe any effect of MPC on the spleen weights of non-infected mice.

In view of the biochemical selectivity of MPC, and its antileukemic effect in animals, MPC has been used clinically to treat childhood leukemia in the terminal state of the disease(Kornhuber and Chandra, 1979; Chandra et al., 1975,1979, 1980; Chandra, 1979).These patients were resistent to all previous chemotherapeutic regimes which involved drugs, such as prednisone, vincristin, daunorubicin, L-asparaginase, Ara-C, 6MP, methotraxate, cyclophosphamid and actinomycin-D. Of the 13 terminal cases (Kornhuber & Chandra, 1979; Chandra et al 1979), complete remission was achieved in 3, and a partial remission was achieved in 2 cases. Fever occasionally accompanied by shivering, was frequently observed under MPC treatment in the first hour after injection. However, these symptoms never lasted more than the first hour, and no other sideeffects could be observed.Differential analysis of cell counts in patients under MPC treatment showed that the cytolysis is restricted to leukemic cells (Kornhuber, Chandra and Welte, unpublished results). Further trials of MPC in childhood leukemia are in progress to delineate the proper conditions for its use. Recent studies from our laboratory have shown that MPC is non-mutagenic (Ames Test, and an in vitro- in vivo test developed in this laboratory; Nashed and Chandra, 1980), does not cause chromosomal aberration in rats, and the drug is tolerated at 100 times the dose used in clinical trials (0.5 mg per kg body weight) by rats, without any noticeable effects.

6. FUTURE PROSPECTS

Viruses can cause malignancy in animals in at least two ways: Firstyl, the expression of the integrated provirus can transform the infected cell, and the progeny of this cell will lead to a tumor.This type of tumor, derived from a single cell, would be a clonal type of tumor. The second situation is, where the infected cell is not transformed to develop a tumor but, it acts as a virus "producer" within the animal. Thus, other cells, not necessarily from a common origin, can get infected and transformed to develop a tumor. This type of tumor would be non-clonal, consisting of "recruited" cells. In this situation, an inhibition of reverse transcriptase will be very valuable as a therapeutical agent. Another interesting situation is the control of remission state. Clinical studies by Fialkow et al.(1971) and Thomas et al. (1972) implicate the presence of infective agents, probably C-type particles, in leukemic patients under remission. Thus , an additional treatment with reverse transcriptase inhibitors may be useful in the remission maintenance therapy.

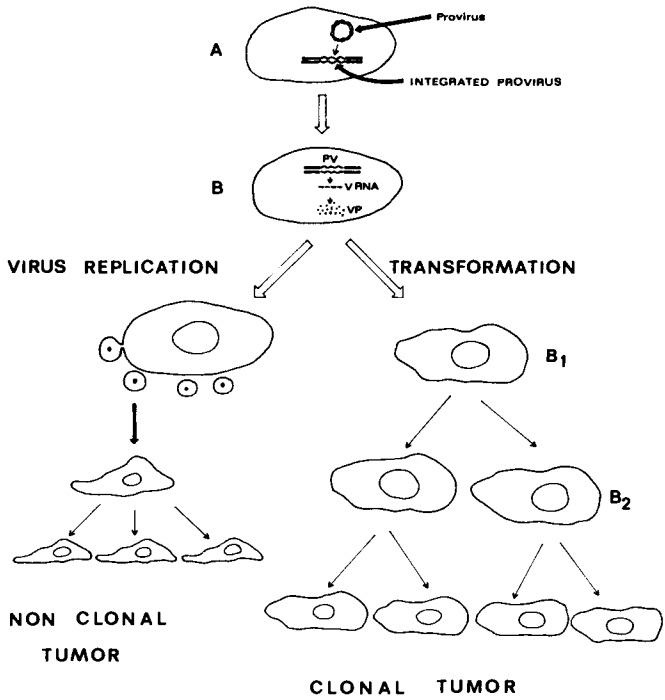


Fig. 19. Possible modes of tumor formation by oncrona-viruses. PV= Proviral DNA; V RNA= Viral RNA; and VP= Viral protein.

ACKNOWLEDGMENTS

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General Mechanisms of Action of Hypolipidemic Agents

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ABSTRACT

The general mechanisms of action of hypolipidemic agents are briefly reviewed, taking as example the known effects of nicotinic acid and clofibrate. The effect of clofibrate on liver peroxysome proliferation is examined and mention is made of the carcinogenic effect of hypolipidemic drugs inducing peroxysome proliferation. The effect of glucagon on the blood lipids is described as illustration of one of the possible mechanisms of hypolipidemia. Newer approaches in the search for anti atherogenic agents are summarized.

KEYWORDS

Nicotinic acid; clofibrate; glucagon; FFA mobilization; lipoprotein synthesis; peroxysome proliferation.

INTRODUCTION

Current interest in hypolipidemic agents is due to the relation between plasma lipid levels and development of coronary heart disease (CHD). Numerous clinical and epidemiological studies have indeed shown, that CHD increases with the plasma lipid levels, particularly that of serum cholesterol. In experimental animals, it has been repeatedly demonstrated that it is possible to produce atherosclerotic lesions by dietary means, and that the development of the lesion is related to the elevation of plasma cholesterol concentration; more specifically, to the concentration of cholesterol transported by the low density lipoprotein (LDL) (Grande, 1979).

It follows from these observations that a reduction of the plasma lipid levels would be a valuable prophylactic measure in the pre-

vention of CHD. Furthermore, because there is indication that experimental atherosclerotic lesions are reversible, reduction of plasma lipid levels would be expected to have also therapeutic value.

But the rationale for hypolipidemic therapy, as noted by Frantz, is a highly controversial topic. In his opinion: How can one weigh the benefits of treatment againts its dangers if one is not sure that the reduction in blood lipids causes any reduction at all in the risk of heart attack and stroke?.

Controlled studies on either secondary or primary prevention of CHD, have casted serious doubts about the usefulness of the hypolipidemic drugs. Thus, the conclusions of the Coronary Drug Project (1975) regarding Clofibrate (Ethyl-alpha, p-chlorophenoxy-isobutyrate) and Nicotinic acid (Niacin, 3-pyridine - carboxylic acid), two widely used hypolipidemic agents, was that the results of the study provided no evidence on which to recommend either clofibrate or nicotinic acid in the treatment of persons with CHD. Moreover, both drugs produced undesirable secondary effects.

The cooperative trial in the primary prevention of ischemic heart disease using clofibrate (1978) led to the following conclusion: "The results of the trial confirm the basic hypothesis that reduction of high serum cholesterol levels, even in middle-age, can reduce the incidence of IHD. However, the fact that clofibrate increases the incidence of gall stones, and the possibility that it may have even more serious local pathological consequences, indicate that it cannot be recommended as a lipid lowering drug for community-wide primary prevention of ischemic heart disease".

On the other hand, one can sympathize with the clinician, who in an effort to help his patients is willing to use hypolipidemic drugs, in spite of the lack of definitive evidence as to their usefulness and safety. In fact, many clinicians believe that drug therapy must be undertaken in all the patients whose blood lipids are not satisfactorily reduced by diet.

The pharmaceutical companies have been very active in producing new hypolipidemic drugs. In 1969, Bencze et al. made a documented survey of some 200 hypolipidemic agents, and two more reviews have been produced by the senior author in 1975 and 1978. In the last of these reviews Bencze is encouraged by the fact that two new drugs (Probucol and Colestipol) have been approved by the U.S.A. Food and Drug Administration, and sounds an optimistic note in view of the regression of atherosclerotic lesions observed in experimental animals and in human patients treated with hypolipidemic drugs.

In contrast, the present reviewer has been impressed by the paucity of reports dealing with hypolipidemic drugs, presented at the European Congress of Cardiology held in Paris in June 1980.

The demonstration of an inverse correlation between the levels of High Density Lipoprotein (HDL) and development of atherosclerosis has added a new dimension to the problem of the relations between plasma lipids, lipoproteins and atherosclerosis. Accordingly, there is obvious interest at the present for the study of the

effect of hypolipidemic drugs on the various lipoprotein fractions, and for the development of drugs capable of increasing HDL concentration (Grande, 1979, Paoletti et al. 1978).

GENERAL MECHANISMS OF ACTION OF HYPOLIPIDEMIC DRUGS

The mechanism of action of most hypolipidemic agents is poorly understood at the present, and there is not a single pattern of action common to all of them. Multiple metabolic effects have been ascribed to different hypolipidemic drugs, but it is difficult to decide which of these effects should be considered as the main responsible for the hypolipidemic action.

On the other hand, it must be realized that hyperlipidemia represents a wide spectrum of metabolic disorders manifested by elevation of the plasma lipid levels, as well as by changes in the proportions of the various lipid fractions and their distribution among the different lipoproteins. A number of hyperlipidemias are genetically determined, whereas others are secondary to endocrine or nutritional imbalances, or other disease processes (Felts and Rudel).

The traditional view in regard to hypocholesterolemic drugs, considers that plasma cholesterol concentration can be reduced by decreasing cholesterol absorption and synthesis and by increasing its catabolism and excretion. This view implies that the changes in total cholesterol content of the body are reflected in changes of the plasma cholesterol concentration, and it has been called by Sodhi et al. the "overload hypothesis".

Because cholesterol is transported by the various lipoproteins, the plasma cholesterol level is determined by the rates of entry into plasma of the lipoproteins synthetized by the liver and the intestine, and by the rates of lipoprotein removal and catabolism. Consequently, plasma cholesterol levels can be reduced by drugs capable of influencing lipoprotein metabolism, without directly affecting the synthesis, absorption and elimination of cholesterol. This view is called by Sodhi et al. the "transport hypothesis". One important feature of this hypothesis is the consideration that the lipid transport mechanism is primarily designed for the transport of the triglycerides; cholesterol itself being a part of the transport mechanism. It is suggested by Sodhi et al. that the plasma cholesterol levels are dictated by the metabolism of the triglycerides, more than by the absorption, synthesis and catabolism of cholesterol itself. In agreement with this view, we have observed that plasma cholesterol levels in dogs are related to the postprandial levels of free fatty acids (FFA) resulting from the lipolysis of circulating chylomicrons (Grande and Prigge, 1974, Prigge and Grande, 1973).

Many of the hypolipidemic drugs currently used are believed to act both upon the synthesis and the transport of the plasma lipids. Two of these drugs: Nicotinic acid and Clofibrate, have been extensively studied clinically and experimentally. A brief review of present knowledge regarding the hypolipidemic action of these two compounds should be helpful for understanding the present situation

in regard to the mechanism of action of hypolipidemic agents.

The hypolipidemic effect of Nicotinic Acid

Twenty five years ago, Altschul et al. observed that the administration of large oral doses of nicotinic acid (3 g per day), was followed by a decrease of serum cholesterol concentration in man. This effect is not produced by nicotinamide which, on the other hand, shares the effects of nicotinic acid as pellagra-preventing vitamin. Moreover, the dose of nicotinic acid required for the reduction of plasma cholesterol level is about 150 times the recommended daily dietary allowance of nicotinic acid. The effect of nicotinic acid on plasma cholesterol depends both on the dose, and the initial plasma cholesterol level. Nicotinic acid did not cause a decrease of plasma cholesterol in individual who had low initial cholesterol levels (Altschul and Hoffer).

There has been some disagreement as to the effect of nicotinic acid on plasma cholesterol in experimental animals (Grande, 1966). In regard to the dog, studies in our laboratory have shown that, for a given dose of nicotinic acid, the cholesterol-depressing effect depends on the initial cholesterol level of the animal. The marked reduction of plasma cholesterol observed in thyroidectomized dogs in our studies, was practically the same as that observed in normal dogs whose plasma cholesterol concentration had been elevated, by feeding coconut oil, to the same level as that of the thyroidectomized dogs. These experiments indicate, therefore, that the presence of the thyroid gland is not required for the cholesterol-depressing effect of nicotinic acid (Grande, 1966).

Early in the 1960's Carlson demonstrated that nicotinic acid has a powerful inhibitory effect on adipose tissue lipolysis, causing a reduction of the plasma levels of circulating FFA. This important discovery provided a useful tool for the pharmacological analysis of the metabolic affects of excessive FFA mobilization (Carlson, 1965, 1978).

Hyperlipidemia due to increased production by the liver of very low density lipoproteins (VLDL), is a well known consequence of excessive FFA mobilization from the adipose tissue. There is overwhelming evidence in support of the view that increased inflow of FFA into the liver causes an elevation of circulating VLDL (Carlson, 1978, Prigge and Grande, 1973).

Accordingly, the plasma cholesterol and triglyceride depressing effects of nicotinic acid are primaryly ascribed to its inhibitory effect of adipose tissue lipolysis.

It seems now clear, however, that the effect of nicotinic acid on the blood lipids can not be solely explained by the inhibition of FFA mobilization from the adipose tissue. Nicotinic acid has a pronounced effect decreasing chylomicron concentration, which cannot be explained by reduced turnover of FFA. According to Carlson (1978), nicotinic acid speeds up the catabolism of triglyceride-rich lipoproteins (chylomicrons included) by increasing lipoprotein lipase activity. At the same time, nicotinic acid increase the rate of fatty acid incorporation into the adipose tissue. Both

these effects of nicotinic acid would be expected to cause a decrease of the circulating triglyceride-rich lipoproteins (chylomicrons and VLDL), without directly affecting the concentration of LDL.

In summary then, it would appear that nicotinic acid causes a decrease of plasma cholesterol and triglycerides by a triple mechanism:

a). Decrease of FFA mobilization, by inhibition of adipose tissue lipolysis.

b). Stimulation of lipoprotein lipase activity.

c). Stimulation of fatty acids incorporation into the adipose tissue triglycerides.

The first of these effects results in a reduction of VLDL synthesis by the liver, whereas the two latter effects speed up the removal of triglyceride-rich lipoproteins from the circulating plasma. The reduction of LDL levels observed in patients treated with nicotinic acid is, however, not caused by an increased catabolism of this lipoprotein, but rather by reduced LDL synthesis consecutive to reduced synthesis of its inmediate precursor, the VLDL (Carlson, 1978).

It seems clear therefore, that the cholesterol-lowering effect of nicotinic acid is not the result of a direct action of the drug on cholesterol metabolism, but the consequence of its effects on FFA mobilization and the metabolism of the triglyceride-rich lipoproteins.

The hypolipidemic effect of Clofibrate and its analogs

Clofibrate, which is particularly effective in lowering plasma triglycerides, has been for many years the most widely used hypolipidemic drug.

The hypolipidemic effect of p-chlorophenoxyisobutyric acid (CPIB) and its ethyl ester (Clofibrate), was first reported by Thorp and Waring in 1962. Since then, a considerable number of phenoxy substituted acids have been synthetized in an effort to produce hypolipidemic analogs more effective than clofibrate, and devoid of undesirable side-effects.

The modifications of the activity profile induced by structural changes of the clofibrate molecule constitute a most interesting exercise in pharmacodynamics which, however, cannot be considered here. The reader is referred to the reviews by Bencze previously mentioned. Reference to the effects of some clofibrate analogs should be made only in so far as they provide insight into the mechanism of action of this important group of hypolipidemic compounds.

Clofibrate and its derivatives have a number of biochemical and metabolic effects; but their basic mechanism of action is unknown at the present. In their studies on the hypolipidemic effects of aryloxybutyrate derivatives, Thorp and Waring noted that the hypolipidemic response to clofibrate in experimental animals was influenced

by thyroid hormone. Clofibrate, and some of its derivatives and analogs, such as methyl clofenapate, are rapidly hydrolyzed in the body to the corresponding acids which, in turn, are bound to and transported by the plasma albumin. It was also found, that aryloxybutyric acids have a very selective competitive effect in regard to the binding of thyroxine to albumin. The British authors developed an in vitro test for assessing the activity of clofibrate derivatives, based on the competitive activity with thyroxine for the same binding sites of albumin. It was postulated that clofibrate, by displacing thyroxine from the plasma to the liver, makes this organ hyperthyroid, while keeping the rest of the body tissues euthyroid.

There are, however, effects of clofibrate which cannot be ascribed to the effect of thyroxine on the liver because, as previously mentioned for nicotinic acid, they are manifested in thyroidectomized animals (Bencze, 1975). Furthermore, certain compounds such as Procetofen (LF-178, Lypanthyl; Isopropyl - [4'-(p-chlorobenzoyl) 2-phenoxy-2-methyl] propionate), which has been shown to be about six times more active than clofibrate shows no correlation between in vitro displacement of thyroxine and hypolipidemic effect in vivo.

Clofibrate brings about a reciprocal shift in the concentrations of VLDL and LDL. As shown by Wilson and Lees, following clofibrate treatment there is a decrease of VLDL cholesterol and an increase in LDL cholesterol. Not all the forms of hyperlipidemia show this response, which is observed mainly in patients of the IIb type. This and other observations (Stäubli and Hess), support the view that clofibrate and other aryloxy acids slow down the process of synthesis, assambley, intracellular translocation, and release of lipoproteins by the liver cells. Clofibrate and other aryloxy acids, reduce the release of lipoproteins by the liver, in the presence of an essentially intact machinery for lipoprotein synthesis and secretion in the liver cell, and without producing fatty liver.

Other studies indicate that, both in normotriglyceridemic and hypertriglyceridemic men, clofibrate lowers plasma triglyceride fatty acids, by improving their clearance in extrasplanchnic tissues (Ryan and Schwartz, Wolfe et al., 1973). Lipoprotein lipase activity in skeletal muscle, adipose tissue and postheparin plasma, have been reported to increase in fasting men during clofibrate administration (Bierman et al., 1970, Lithell et al., 1978, Nikkila et al., 1977, Taylor et al., 1977).

Recent experiments by Wolfe et al. (1980) have shown that in the glucose-fed state, clofibrate markedly decreases splanchnic (hepatic) triglyceride secretion and alters the splanchnic exchange of lactate, glycerol and amino acids. Administration of clofibrate to hypertriglyceridemic subjects maintained on a high carbohydrate diet for 2 weeks, and receiving a prolonged infusion of glucose (30 g/hour) and labeled palmitic acid, caused plasma triglyceride fatty acids 35 percent lower than observed control values. Splanchnic secretion of triglycerides was 68 percent lower in clofibrate treated subjects than in controls. Systemic transport and splanchnic uptake and conversion of free fatty acids to plasma triglycerides were unaltered by clofibrate. The mean fraction of

triglyceride fatty acids of plasma VLDL derived from precursors other than free fatty acids, however, was significantly lower in subjects receiving clofibrate than in controls (73 vs 91 percent).

These studies indicate that the inhibition by clofibrate of the rise in plasma triglyceride levels ocurring during the second week of ingesting a high carbohydrate diet, is attributable to decreased splanchnic (hepatic) secretion of triglyceride fatty acids. In the glucose-fed state there is no evidence of reduced FFA transport or conversion to triglyceride fatty acids. Under the conditions of these experiments therefore, clofibrate reduces hepatic synthesis of glycerolipids from precursors other than FFA, but not from FFA. These results are in agreement with observations showing that clofibrate reduces glycerolipid synthesis in fed rats by inhibiting glycerol-3-phosphate acyl transferase activity (Wolfe et al. 1980).

In summary, it would appear that the triglyceride-lowering effect of clofibrate, in the glucose-fed state is mainly attributable to inhibition of hepatic secretion of triglycerides, consecutive to reduced synthesis from precursors other than FFA. Clofibrate does not appear to enhance extrasplanchnic removal of triglyceride fatty acids in the glucose-fed state.

Hypolipidemic drugs as hepatic peroxysome proliferators

One of the most remarkable effects of clofibrate in experimental animals consists in the development of massive hepatomegaly, associated with a marked increase of peroxysomes in the liver parenchymal cells. Following Paget's original observations, numerous publications, which have been reviewed among others by Stäubli and Hess, have been devoted to this effect, and should not be analyzed here.

Peroxysomes are ubiquitous cytoplasmic organelles which contain catalase and other hydrogen peroxyde producing oxydases, carnitine acetyltransferase, and enzymes involved in the beta-oxidation of long-chain fatty acids. The activities of these enzymes in the liver tissue are elevated in association with peroxysome proliferation, but the catalase activity does not increase in proportion to the volumetric increase of the peroxysome compartment. This effect has been demonstrated in three rodent species (rats, mice and hamster) for clofibrate and other hypolipidemic agents (Reddy et al. 1980), and it has been demonstrated that hepatomegaly and peroxysome proliferation persist as long as the drug is administered, disappearing upon its withdrawal.

Among the hypolipidemic drugs inducing peroxysome proliferation are the following:

1. Clofibrate: Ethyl, alpha-p-chlorophenoxyisobutyrate.

2. Procetofen: Isopropyl- [4'- (p-chlorobenzoyl)-2-phenoxy-2-methyl] propionate.

3. Tiadenol: Bis - (hydroxyethylthiol) 1-10 decane.

4. Gemfibrozil: 5- (2,5-dimethylphenoxy) 2,2-dimethyl pentanoic acid.

5. Nafenopin: 2-methyl-2- [p-(1,2,3,4 tetrahydro-1-naphtyl) phenoxy] propionic acid.

6. Wy 14643: [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid.

7. Br 931: 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio (NB-hydroxyethyl) acetamide.

8. Tibric acid: 2-chloro-5-(3,5-dimethyl piperidinosulphonyl) benzoic acid.

Compounds 2, 3 and 4 (Procetofen, Tiadenol, Gemfibrozil), have been recently reported to cause a dose-related increase of liver weight, when fed for 4 weeks to male F-344 rats, at dietary levels of 0,05, 0,1 and 0,2 percent. Hepatomegalic effect was greater for compounds 2 and 3 than for compound 4. All three compounds caused peroxysome proliferation, associated with marked increase of hepatic catalase and carnitine acetyltransferase activities (Reddy, 1980).

These intriguing observations should be of considerable interest for the eventual understanding of the mechanism of action of the hypolipidemic drugs. However, as far as is known to the present reviewer, the relation between the biochemical changes associated with peroxysome proliferation and the mechanism of hypolipidemic action is still obscure.

On the other hand, it is important to note that compounds 1, 5, 6, 7 and 8 of the preceding list, when chronically fed, induced a high incidence of hepatocellular carcinomas in mice and/or rats. In view of the development of liver tumours in animals fed these structurally different hypolipidemic compounds, it has been postulated that hypolipidemic, hepatic peroxysome proliferator agents form a novel class of chemical carcinogens (Reddy et al. 1980).

There is no need to insist here about the obvious importante of these observations for the clinical use of such hypolipidemic agents.

THE HYPOLIPIDEMIC EFFECT OF GLUCAGON

In 1962 Amatuzio, Grande and Wada found that administration of glucagon (1,0 to 5,0 mg daily) over a period of several days, caused a reduction of the blood lipids in some hyperlipidemic patients. More recently, Aubry et al. confirmed and extended this observation in a larger group of hyperlipidemic patients of various types. The Canadian authors noted that glucagon, at the relatively side-effect free level of 3 mg/day, cannot be considered as a valuable cholesterol-lowering agent; and this opinion seems to be perfectly justified.

The present comment on the hypolipidemic effect of glucagon is included here, not because of the clinical usefulness of this hormone in the treatment of hyperlipidemia, but rather as an illustration

of the factors involved in the mechanism of hypolipidemic action.

In a comparative study of the effects of glucagon on FFA mobilization carried out in our laboratory (Grande, 1976), it was shown that this hormone causes fatty liver in birds (Grande and Prigge, 1970), and a decrease in the secretion of triglycerides by the liver (De Oya et al.). It was suggested that this effect is related to a decrease in the synthesis of the apolipoprotein moiety of the VLDL. A similar suggestion was made by Eaton, who observed that glucagon has a hypolipidemic effect in rats with hyperlipidemia induced by treatment with Co. In these animals, the administration of glucagon inhibited the incorporation of labeled aminoacids into the lipoproteins's apoprotein, with reduction of circulating VLDL, triglycerides and the pre-beta band.

These observations indicate that it is possible to reduce the plasma lipid levels by interfering with the synthesis of lipoprotein apoproteins by the liver; but this mechanism involves the risk of production of fatty liver.

NEW APPROACHES TO HYPOLIPIDEMIC AGENTS

Structural modification of preexisting hypolipidemic compounds, in an effort to increase their activity and to avoid harmful side-effects, is still an active field of research. New compounds structurally different from the original hypolipidemic drugs have been also developed and are used in the clinic.

The disappointing results of the preventive trials mentioned at the beginning, and new insights on the mechanism of the hypolipidemic effect, have led to the search for new approaches in the treatment of atherosclerosis, including the development of drugs capable of preventing the development of the atherosclerotic lesion. Reduction of plasma cholesterol and other blood lipids is not the primary goal in this approach. The main objective is to reduce the atherogenic activity of the circulating lipoproteins. To the extent that some of these agents have little effect on the plasma lipid concentration, they do not properly qualify as hypolipidemic agents. Nevertheless, I believe that a brief mention of these new approaches would be an appropiate end to this review.

Among others, the following approaches attract much current attention:

a). Modification of lipoprotein metabolism, in order to increase the ratio of HDL to VLDL + LDL circulating lipoproteins.

b). Assay of substances capable of affecting the lipoprotein uptake by the arterial wall.

c). Modification of the apoprotein component of the circulating lipoproteins, in order to decrease their atherogenic power.

Increasing knowledge about the structure and metabolism of the plasma lipoproteins and their role in atherogenesis, shall be a decisive factor in the development of the new approaches to the pharmacological prevention and teatment of atherosclerosis just outlined.

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Central Mechanisms of Anorectic Drugs

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ABSTRACT

Anorectics can be divided on the basis of their mechanism of action into those like amphetamine and other phenylethylamines which depress food intake by interacting with brain catecholamines, and those such as fenfluramine and some piperazine derivatives for which serotonin plays a major role. This second class is of particular interest since food intake is reduced by doses causing no signs of central stimulation. Recent data on their neuronal mechanisms of action will be discussed. This new information may be useful for developing drugs which selectively depress appetite.

KEYWORDS

Amphetamine; mazindol; diethylpropion; fenfluramine; quipazine; m-chlorophenylpiperazine; anorexia; serotonin; catecholamines.

INTRODUCTION

There is considerable evidence that brain catecholamines and serotonin mediate the anorectic activity of different drugs. These brain substances are also involved in drug effects other than depression of feeding, such as locomotor stimulation, stereotyped movements or sedation, depending on what drug and what dose is used. There is no general agreement on the exact role of each brain amine in the regulation of feeding or mediation of drug effects.

This article examines the type of interaction of anorectics with brain dopamine (DA), noradrenaline (NA) and serotonin (5HT) and the role of these substances in drug activity, in an attempt to demonstrate that:

1) the behavioral stimulation and reinforcing properties of amphetamine, mazindol and, to a lesser degree, diethylpropion depend on their ability to release DA from nerve endings and inhibit its reuptake into the neuron. In spite of various suggestions of dopamine involvement in feeding, its role in drug-induced anorexia is questionable;

2) the anorectic activity of amphetamine, mazindol and diethylproprion is due

mainly, if not exclusively, to their ability to release noradrenaline (and/or adrenaline) from central adrenergic neurons associated with the so-called ventral noradrenergic bundle (VNB). This system appears to be specifically involved in suppression of feeding since the motor stimulatory effects of these compounds are not prevented by destruction of this area. Some aspects of amphetamine's stimulatory action may involve noradrenergic neurons other than those of the VNB;

3) fenfluramine and some piperazine derivatives such as quipazine and m-chlorophenylpiperazine (mCPP) depress feeding by releasing serotonin from central neurons and/or mimicking its action on postsynaptic receptors. These drugs have little effect on catecholamines or act upon them differently from amphetamine. The lack of central stimulatory effects may be due to their inability to activate catecholaminergic mechanisms in the brain. In discussing these topics three types of evidence will be considered:

1) in vitro studies show that anorectics affect monoamine membrane uptake, neuronal release and receptor activity, mechanisms considered of particular importance for neurotransmission in intact animals.

2) Anorectics cause changes in the monoamine metabolism in the brain which are consistent with the effects observed in vitro. Knowledge of the utility and limitations of amine metabolism changes in revealing functional activity and of the existence of functional interaction between monoamines in the brain is a prerequisite for appropriate interpretation of the results.

3) Procedures affecting brain monoamine mechanisms alter the activity of anorectics. This is a crucial aspect for defining the functional role of amines in drug activity since biochemical studies cannot provide information on the functional output of drug effects on monoamine mechanisms.

For the sake of convenience, DA, NA and 5HT will be discussed separately, although obviously they are not functionally isolated, and anorectics can act on more than one monoamine at a time in the brain.

DOPAMINE

Biochemical Effects

D-amphetamine markedly inhibits the uptake of dopamine and releases it from synaptosomes prepared from rat brain (Garattini and Samanin, 1976). This last effect is particularly evident in synaptosomes from animals treated with reserpine (Farnebo, 1971). Since this drug depletes the neurons of most of the stored amines (Brodie and others, 1957), the data indicate that amphetamine preferentially releases dopamine from a small reserpine-insensitive pool. The functional importance of this mechanism is shown by the fact that the central stimulation induced by low doses of amphetamine, which depends on the drug's effect on brain dopamine, is either not affected or may even be increased by reserpine treatment (Svensson, 1970) while it is abolished by inhibition of dopamine synthesis (Weissman and others, 1966), on which the functional pool appears to depend directly. Amphetamine does not seem to act directly on central dopamine receptors, as it does not significantly displace the binding of ligands for dopamine receptor sites in brain membranes (Burt and others, 1976). Consistent with the increase of dopamine release caused by amphetamine in vitro is its ability, when given at high doses (Garattini and others, 1975a) to increase striatal levels of homovanillic acid (HVA), one main metabolite of DA. At low anorectic doses, however, amphetamine can reduce HVA concentrations in the brain (Garattini and others, 1978).

This biphasic effect on dopamine metabolism observed at different doses is not particularly surprising considering the preferential action of low concentrations on the small functional pool. Release from this pool in fact would make more amine available for receptors without exposing too much amine to catabolizing enzymes, thus favouring dopamine metabolism reduction consequent to a feedback mechanism following receptor stimulation.

This is well documented with drugs that mimic the action of dopamine on post-synaptic receptors (Roos, 1969; Garattini and others, 1975b). Mazindol is a powerful inhibitor of DA uptake whereas diethylpropion is much less active (Garattini and others, 1978). These drugs are weaker DA releasers than d-amphetamine in synaptosome preparations (Offermeier and du Preez, 1978) but at high doses they markedly increase striatal levels of HVA (Garattini and others, 1975a). The scant effect of diethylpropion on dopamine mechanisms in vitro may depend on the fact that its actions are mainly mediated by the formation of active metabolites in the organism (Beckett, 1979). At anorectic doses, however, this drug produces less signs of dopaminergic activation than amphetamine and does not change the levels of striatal HVA (Garattini and others, 1978) suggesting it is less able to increase dopaminergic function.

Mazindol, instead, causes an increase of brain DA metabolism even at anorectic doses (Garattini and others, 1978), a finding which would agree with the suggestion that this drug may release dopamine by a mechanism (reserpine-sensitive pool) different from that used by amphetamine (Offermeier and du Preez, 1978). The same suggestion has been made for diethylpropion (Offermeier and du Preez, 1978).

As regards effects on DA receptors, no information is available, to our knowledge, on the ability of mazindol and diethylpropion to displace dopamine receptor binding to rat membranes.

Fenfluramine shows little effects on presynaptic DA mechanisms with the possible exception of the l-isomer which may cause intraneuronal release of dopamine (Bendotti and others, 1980). At doses higher than those causing reduction of food intake, fenfluramine raises HVA levels in the striatum of rats (Garattini and others, 1975a). Since this effect is prevented by direct dopamine agonists such as apomorphine and piribedil, it was suggested that fenfluramine acts on brain dopamine through a mechanism similar to that of neuroleptics (Jori and others, 1974). Further studies however have shown that, unlike neuroleptics, fenfluramine does not displace dopamine ligand binding to brain membranes (Burt and others, 1976), indicating that mechanisms other than postsynaptic receptor blockade may be responsible for the changes of dopamine metabolism. It has been recently suggested (Crunelli and others, 1980) that the effect of the d-isomer of fenfluramine on striatal dopamine metabolism is mediated by its ability to activate serotoninergic function in the brain since the effect is blocked by drugs which prevent fenfluramine's action on serotonin. This however does not apply to the l-isomer whose effects on dopamine metabolism are not affected by drugs acting on serotonin (Crunelli and others, 1980). Considering that in vivo d and l fenfluramine form their respective deethylated metabolites which also act on serotonin and dopamine (Garattini and others, 1979), the exact mechanism by which fenfluramine interacts with the dopaminergic system is difficult to define. Reduction of dopaminergic activity appears to be the overall effect of fenfluramine as shown by functional studies (Bendotti and others, 1980).

Quipazine and mCPP are poor uptake inhibitors and releasers of DA (Garattini and others, 1978; Samanin and others, 1979), and no information is available on their effects on dopamine receptors. At high doses they can increase the metabolism of brain dopamine but, as in the case of d-fenfluramine, this effect may be mediated by their increasing central serotoninergic activity (Samanin, unpublished results).

Functional Results

Various findings suggest that the locomotor stimulation and stereotyped movements caused by amphetamine in several animal species are mediated by its effects on brain dopamine. In fact, selective destruction of central dopaminergic neurons or treatment with DA antagonists prevents amphetamine affecting motor behavior (Creese and Iversen, 1973; Fibiger and others, 1973). The overstimulation resulting from increased dopaminergic function has made it difficult to assess whether dopamine is involved in amphetamine anorexia as well. The main difficulty lies in the fact that maximal anorexia is usually obtained in animals with doses causing stereotyped movements, which obviously interfere with eating. That DA antagonists completely inhibit the effect of amphetamine on motor behavior while only partially counteracting the depression of feeding favors this interpretation (Samanin and others, 1978; Quattrone and others, 1977). Difficulties in interpreting changes of dopaminergic activity in relation to feeding behavior also arise from findings that relatively low doses of apomorphine, a dopamine agonist, either enhance or depress feeding depending on whether the animals are satiated or food deprived respectively (Eichler and Antelman, 1977). This, with other findings (Marshall and others, 1974), suggest that changes in feeding noted when dopamine activity changes are in all probability due to changes in behavioral arousal and sensory-motor activation rather than an effect on mechanisms specifically related to feeding. In spite of various suggestions (Leibowitz and Rossakis, 1978; Ungerstedt, 1971), no convincing evidence has yet been provided that dopamine is specifically involved in feeding regulation.

In discussing the role of dopamine in amphetamine action it should be mentioned that this amine has been implicated in central motivational and/or reinforcement processes as well as in the reinforcing properties shown by amphetamine in various species (Fibiger and Phillips, 1979). Dopamine appears to be involved in the euphoric effects and addictive properties of amphetamine in humans too since dopamine receptor blockers markedly reduce them (Gunne, 1977). Amphetamine anorexia in humans, however, is not affected by pimozide (Silverstone, 1978) thus disproving dopamine's involvement in this effect.

Mazindol and diethylpropion, like amphetamine, cause central stimulation (Garattini and others, 1978; Garattini and Samanin, 1976). The effect depends on central dopaminergic mechanisms since it is blocked by treatment with dopamine receptor blockers (Carruba and others, 1978; Offermeier and du Preez, 1978; Borsini and others, 1979; Garattini and Samanin, 1976). The effects of mazindol and diethylpropion on motor behavior are blocked by drugs interfering with the synthesis and storage of brain dopamine (Carruba and others, 1978; Offermeier and du Preez, 1978) indicating that presynaptic dopamine mechanisms are mainly involved. As regards food intake, the effect of mazindol, but not that of diethylpropion, is partially prevented by procedures which reduce central dopaminergic activity (Borsini and others, 1979; Samanin and others, 1977a). Mazindol, however, is more potent than diethylpropion on dopaminergic mechanisms and motor behavior (Garattini and others, 1978). Thus, as in the case of amphetamine, excessive motor stimulation may partially be responsible for the blockade of feeding caused by this compound in rats.

Although mazindol has a chemical structure different from that of phenylethylamine, it appears to be as potent as amphetamine in increasing dopaminergic activity whereas diethylpropion, a true phenylethylamine, appears to be less active in this respect. This may explain the less addictive properties of diethylpropion in man (Hoekenga and others, 1978). At anorectic doses, fenfluramine does not cause motor stimulation or stereotyped movements (Garattini and others, 1978) but it does block the stimulatory effects of amphetamine (Bendotti and others, 1980). Since amphetamine's effects on motor behavior are mainly mediated by increased released

of brain dopamine, these findings suggest that fenfluramine reduces the activity of presynaptic dopaminergic neurons in the brain, probably through its action on brain serotonin. There is evidence in fact that serotonin in the brain may normally inhibit dopaminergic activity (Costall and Naylor, 1978) and the slight sedation caused by fenfluramine may be due to the indirect effect of the d-isomer on dopamine. The limited tendency among patients to abuse fenfluramine (Estrada, 1979; Götestam, 1979) may also partially depend on the drug's inability to cause central dopaminergic activation. Signs of central dopaminergic activation have been noted by some authors in animals treated with quipazine (Grabowska and others, 1974) but no such effects have been reported with mCPP (Samanin and others, 1979). The mechanism by which quipazine increases dopamine activity in some instances is not known.

NORADRENALINE

Biochemical Effects

D-amphetamine is a powerful inhibitor of noradrenaline uptake in synaptosomal preparations (Garattini and others, 1978). As regards its ability to release noradrenaline from nerve endings the evidence is more confusing since either no effect or an enhanced NA release have been found in in vitro preparations treated with amphetamine (Raiteri and others, 1975; Ziance and others, 1972). The apparent discrepancies are probably due to the inherent difficulties in studying noradrenaline release in vitro, since most in vivo biochemical and functional studies suggest that amphetamine does cause release of noradrenaline (Garattini and others, 1975a; Garattini and Samanin, 1976). This is clear from the fact that d-amphetamine significantly increases the metabolism and turnover of brain noradrenaline (Garattini and Samanin, 1976). d-Amphetamine does not seem to act significantly on beta or alpha adrenergic postsynaptic receptors (Bylund and Snyder, 1976; U'Prichard and others, 1977). This is also indicated by the fact that integrity of presynaptic noradrenergic neurons is a prerequisite for its anorectic activity (Samanin and others, 1975; 1977a).

Like d-amphetamine, mazindol and diethylpropion activate central noradrenergic mechanisms (Garattini and others, 1975a; Offermeier and du Preez, 1978) whereas fenfluramine releases noradrenaline mainly at the intraneuronal level with most amine catabolized by monoamine oxidases before leaving the nerve terminals (Ziance and Rutledge, 1972). It cannot be excluded however that to some extent fenfluramine activates adrenergic mechanisms in intact animals, since it has been described as having sympathetic effects (Lake and others, 1979). Quipazine and mCPP have shown no particularly significant effects on NA uptake and release or receptor binding to brain membranes (Garattini and others, 1978; Samanin and others, 1979; Samanin, unpublished results).

Functional Effects

At low doses amphetamine increases spontaneous locomotor activity and many behavioral performances and these effects may be partially mediated by its effect on noradrenaline (Maj and others, 1972; Schoot van der and others, 1962). Depression of feeding is however common after moderate doses of amphetamine (about 1-2 mg/kg in rats). It has been suggested that amphetamine depresses feeding by activating beta-adrenergic and dopaminergic sites in the perifornical area of the hypothalamus (Leibowitz and Rossakis, 1978) because the reduction of food intake caused by local application of amphetamine in this area is prevented by dopamine and beta-adrenergic blockers (Leibowitz, 1978). Amphetamine effects

however were obtained after injection of 50-100 nmoles of the drug, much more than that found in the whole brain after systemic injection of a dose causing maximal anorexia (Jori and others, 1978); this casts doubt on the significance of these findings in interpreting the action of systematically administered amphetamine. That central noradrenergic neurons are involved in the anorectic activity of amphetamine is suggested by the fact that selective lesions of noradrenergic fibers passing through the so-called ventral noradrenergic bundle (VNB) completely prevent amphetamine affecting food intake (Ahlskog, 1974; Borsini and others, 1979; Samanin and others, 1977a).

Of interest is the fact that this lesion does not prevent the drug acting on motor behavior (Quattrone and others, 1977) arguing in favor of a specific role of this system in amphetamine anorexia. These findings disprove the assumption that amphetamine anorexia is closely associated with its stimulatory effects.

Some authors (Cox and Maickel, 1975) doubt that amphetamine anorexia is mediated by central noradrenergic mechanisms since treatment with alpha-methylpara-tyrosine (alpha MpT), a blocker of catecholamine synthesis, completely prevents the stimulatory action with little effect on the reduction of food intake. In addition, dopamine beta hydroxylase inhibitors, which selectively block noradrenaline synthesis, hardly affect amphetamine anorexia (Franklin and Herberg, 1977), thus also apparently confuting noradrenaline involvement. It should however be considered that, while amphetamine-induced release of dopamine closely depends on synthesis of the amine (Van Rossum and others, 1962), the mechanism of noradrenaline release appears to involve removal of the amine from a larger pool less dependent on amine synthesis (Engberg and Svensson, 1979). This difference is illustrated by the fact that reserpine, which preferentially affects catecholamine storage, significantly counteracts the anorectic activity but not the stimulatory effects of amphetamine (Schmitt, 1973; Neill and Grossman, 1971). Amphetamine at relatively high doses causes marked depletion of brain noradrenaline with little effect on dopamine (Garattini and others, 1975a), in agreement with this hypothesis. Thus, the data with alpha MpT and dopamine beta-hydroxylase inhibitors do not disprove that noradrenaline mediates the effect of amphetamine on food intake.

It has been found that adrenaline-containing fibers run with noradrenergic fibers in the VNB (Hökfelt and others, 1974) and adrenaline depresses feeding when injected in the perifornical area (Leibowitz and Rossakis, 1978). Since most procedures and drugs used to affect noradrenergic mechanisms also act on brain adrenaline, the relative roles of these brain amines in amphetamine anorexia are difficult to establish.

The locus coeruleus is not involved in the depression of food intake caused by amphetamine in rats (Quattrone and others, 1977) since lesions of this area do not modify amphetamine anorexia. In view of the fact that it is involved in the mechanism of arousal and wakefulness (Jouvet, 1974; Fuxe and others, 1974) and that noradrenergic neurons arising in this area facilitate dopaminergic function in the brain (Pycock et al., 1975; Andén and Grabowska, 1976), it is more likely that part of the stimulatory effects of amphetamine depend on its ability to release noradrenaline from terminals of neurons originating in this brain area.

Although some authors favor an involvement of brain dopamine in the anorectic activity of mazindol (Carruba and others, 1978), the effects on noradrenaline appear to be necessary for mazindol and diethylpropion to depress food intake as shown by the fact that lesions of the VNB completely prevent the anorectic activity of these drugs (Borsini and others, 1979; Samanin and others, 1977a). VNB lesions do not prevent the reduction of food intake caused by fenfluramine, quipazine and mCPP (Garattini and Samanin, 1976; Samanin and others, 1979), showing

that neurons of this system are not involved in their anorectic activity.

SEROTONIN

Biochemical effects

Fenfluramine releases serotonin from nerve endings and inhibits its reuptake into the neuron (Garattini and Samanin, 1976; Garattini and others, 1975b). The mechanism by which it releases 5HT may differ according to whether the parent drug or its main metabolite, norfenfluramine, is given. Reserpine pretreatment prevents the effect of d-fenfluramine (dF) on 5HT release but potentiates the effect of d-norfenfluramine (dNF) (Mennini and others, 1980) suggesting that these compounds normally release 5HT from two different pools. The effects of l-fenfluramine and l-norfenfluramine are less altered by reserpine treatment (Mennini and others, 1980). Mazindol is a potent inhibitor of serotonin uptake (Garattini and others, 1978) while diethylpropion and amphetamine have little effect on this mechanism. d-Amphetamine, mazindol and diethylpropion are poor releasers of 5HT in in vitro preparations (Offermeier and du Preez, 1978). Quipazine and mCPP are less active than fenfluramine in releasing 5HT from synaptosomes of normal rats but their effect is markedly potentiated by reserpine treatment (Mennini and others, 1980). Quipazine is as potent as d-fenfluramine in inhibiting 5HT uptake whereas mCPP is less active in this respect (Samanin and others, 1980). As regards the drug's ability to mimic serotonin's action on postsynaptic receptors, it has been found that mCPP is a potent displacer of ^{3}H-5HT binding to rat brain membranes (Samanin and others, 1979), quipazine possesses some affinity for 5HT receptor binding sites (Samanin and others, 1980) and amphetamine and fenfluramine have only scant effect on ^{3}H-5HT binding (Bennett and Snyder, 1976; Garattini and others, 1979). No information is available on the effect of mazindol and diethylpropion in this respect.

Fenfluramine, quipazine and mCPP cause a significant reduction of serotonin metabolism in the brain (Garattini and others, 1975a; Samanin and others, 1977b, 1979). This effect is best explained as the result of a feedback mechanism following 5HT receptor activation induced by these drugs. Unlike quipazine and mCPP, which either do not affect or tend to increase brain concentrations of 5HT, fenfluramine causes marked depletion of brain serotonin (Garattini and others, 1975a). The intimate mechanism of the 5HT depletion by fenfluramine is not clear; interference with the storage mechanism for 5HT (particularly relevant for d-fenfluramine) together with marked activation of 5HT receptors can play a major role. At high doses amphetamine can increase the synthesis of brain serotonin (Reid, 1970) but it is not clear whether this effect is direct or secondary to other actions in the central nervous system. At anorectic doses d-amphetamine, mazindol and diethylpropion do not modify brain levels of 5HT or 5-hydroxy-indolacetic acid (5HIAA) in rats (Garattini and others, 1978).

Functional Studies

The following findings suggest that serotonin is involved in the anorectic activity of fenfluramine:
a) destruction of 5HT neurons prevents fenfluramine anorexia (Samanin and others, 1972; Clineschmidt and others, 1974);
b) fenfluramine anorexia is counteracted by treatment with 5HT antagonists (Garattini and Samanin, 1976);
c) inhibitors of 5HT uptake mechanism which prevent the effect of fenfluramine on brain serotonin significantly reduce its effect on food intake (Garattini and Samanin, 1976; Garattini and others, 1974).

Although some authors have found a reduction of fenfluramine anorexia in animals injected intracerebrally with 5,6 dihydroxytryptamine (Clineschimdt and others, 1974) which is neurotoxic for central 5HT neurons (Nobin and Björklund, 1978) other authors using 5,7-dihydroxytryptamine (5,7 HT), which also causes depletion of brain 5HT (Nobin and Björklund, 1978) found either no effect or even a potentiation of fenfluramine's effect on food intake (Hoebel and others, 1978). These authors suggest that fenfluramine acts at postsynaptic 5HT receptors or through mechanisms other than 5HT. The first hypothesis is not likely since, as we have seen, fenfluramine is a very poor displacer of ^{3}H-5HT binding at brain membranes. In view of the ample evidence of 5HT's involvement in fenfluramine anorexia, a possible explanation of 5,7 HT's failure to affect fenfluramine anorexia in rats is its relative effectiveness in causing 5HT depletion in certain brain areas (Nobin and Björklund, 1978). The integrity of an area close to the pons-mesencephalon raphe nuclei has been shown to be crucial for the anorectic activity of fenfluramine in rats (Samanin and others, 1972) and the brainstem is particularly resistant to the action of 5,7HT on serotonin (Nobin and Björklund, 1978).

Another serotonin-dependent effect of fenfluramine is its ability to reduce food intake by sparing protein consumption (Wurtman and Wurtman, 1977), as shown by the fact that drugs enhancing 5HT transmission reduce carbohydrate consumption (Wurtman and Wurtman, 1979) while depletion of brain 5HT selectively reduces protein intake in rats (Ashley and others, 1979). Amphetamine on the other hand reduces protein and calory intake proportionally (Wurtman and Wurtman, 1977).

Another interesting difference between these drugs is that fenfluramine blocks hyperphagia induced in rats by tail pinch, whereas amphetamine has no such effect (Antelman and others, 1979). This effect of fenfluramine appears to be mediated by its action on 5HT since it is also observed with 5HT agonists (Antelman and others, 1979). These findings are of interest in view of the suggested similarities between hyperphagia induced by tail pinch stress in rats and some forms of excessive eating in humans (Antelman and others, 1979).

On the basis of some differences noted between fenfluramine and amphetamine in changing particular aspects of feeding such as onset of feeding, meal size, rate of eating, etc., it has been suggested (Blundell and others, 1976) that fenfluramine is primarily involved in satiety mechanisms while amphetamine acts mainly by reducing hunger. However no convincing evidence has been provided in support of this hypothesis and some of the different effects of the two drugs may well be explained by their different kinetics and/or general effects on behavior (sedation versus stimulation). Lesions of central serotoninergic neurons or 5HT antagonists do not prevent the effect of d-amphetamine, mazindol and diethylpropion (Samanin and others, 1977a; Borsini and others, 1979) on food intake, arguing against a role of 5HT in anorexia caused by these drugs.

Considerable evidence suggests that quipazine and mCPP depress feeding by increasing central serotoninergic activity (Samanin and others, 1977b, 1979). Part of quipazine's effect on feeding may derive from its ability to release serotonin from nerve terminals since lesions of central serotoninergic neurons partially prevent its reducing food intake (Samanin and others, 1978), while mCPP's ability to mimic serotonin at postsynaptic receptors is considered of major importance for its anorectic activity (Samanin and others, 1979). Drugs or lesions interfering with catecholamines do not modify the effects of quipazine or mCPP (Samanin and others, 1977b, 1979). Another piperazine derivative has been recently shown to depress feeding in rats and cats (Clineschmidt and others, 1977). Since this drug shows no affinity for serotonin binding sites in vitro (Fuller and others, 1978) it may depress feeding by a presynaptic mechanism. This drug, like d-norfenfluramine, releases serotonin from a reserpine-insensitive pool (Mennini and others, 1980). Recent studies on the mechanism by which feeding is depressed by

drugs acting on serotonin show that increased serotonin release and/or direct stimulation of postsynaptic receptors are more important in anorexia than other mechanisms such as uptake inhibition (Samanin and others, 1980). This is clearly shown by the fact that a new potent and selective inhibitor of 5HT uptake in the brain, 4-(3-indolyl-2 ethyl) piperidine (LM 5008), does not significantly depress feeding in rats at a dose 27 times the ED_{50} for inhibiting 5HT uptake in vivo (Samanin and others, 1980).

CONCLUSIONS

Although brain monoamines are not likely to play an exclusive role in feeding regulation (and for some of them a physiological role in feeding may be questionable), they are certainly involved in the reduction of food intake observed with most anorectics so far available. Knowledge of their mechanisms and the way to act upon them appears to offer the best possibilities for pharmacologically controlling feeding excesses. In developing new anorectics acting on brain monoamines the following points are worth careful consideration:

1) Apart from the doubts about dopamine's role in feeding, the involvement of this amine in sensory-motor and central reinforciment processes makes it unlikely that drugs acting on this amine will be of any use in controlling excessive eating and obesity;

2) The development of drugs acting on noradrenaline appears to be more promising. To minimize peripheral actions or central effects not related to feeding, drugs with high affinity for central adrenergic receptors more specifically involved in feeding, such as those innervated by the ventral noradrenergic bundle, should be developed. But this strategy has still to be designed;

3) Although it is not yet clear to what extent feeding can be selectively affected by changing central serotonin transmission, drugs of this class present the enormous advantage of depressing feeding without causing central stimulation in animals and man. Development of and extensive studies with drugs releasing 5HT or mimicking its action in the central nervous system appear to be a very promising area for future research.

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Antiobesity Agents Acting Through Peripheral Mechanisms

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ABSTRACT

Antiobesity agents which function through peripheral mechanisms are reviewed. Areas in which these agents may regulate energy metabolism include modulators of appetite regulation, nutrient absorption, lipid metabolism and endocrine function.

The gastrointestinal peptide hormones, cholecystokinin and bombesin have satiety producing properties. These effects have encouraged the search for analogs. Two novel non-peptide anorectic agents, (±)-trans-epoxyaconitic acid and (−)-threo-chlorocitric acid appear to function through gastrointestinal mechanisms.

Selective reduction in the absorption of nutrients has been achieved. Decreased carbohydrate absorption by the glucosidase inhibitor, Bay g 5421, has been effective in rodents and man. Decreased lipid absorption by Pluronic L-101 exhibited antiobesity effects in rodents.

Inhibitors of lipid synthesis have exhibited antiobesity activity in rodents: (−)-hydroxycitrate, an inhibitor of citrate cleavage enzyme; dehydroepiandrosterone, an inhibitor of glucose-6-phosphate dehydrogenase; and nafenopin, an inhibitor of acetyl CoA carboxylase. Decreased efficiency of oxidation has been achieved by thyroid hormone administration and by uncouplers of oxidative phosphorylation. Agents which enhance lipid mobilization are sought as antiobesity drugs.

Normalization of hormone imbalances as a means of treating obesity is reviewed. Agents which decrease the hyperinsulinemia associated with obesity are oxytetracycline, somatostatin analogs, pancreatic peptides, and l-dopa.

KEY WORDS

Antiobesity agents; peripheral mechanisms; anorectic peptides; anorectics; intestinal absorption; lipid synthesis inhibitors; enhanced lipolysis; enhanced lipid oxidation; hormone normalization.

INTRODUCTION

The magnitude of the obesity problem and the relative ineffectiveness of current treatment modalities have encouraged investigations of other pharmacological approaches. In addition, the limitations of some of the currently available appetite suppressants, particularly the development of tolerance and stimulant side effects, have provided the impetus for the exploration of novel pharmacological approaches to obesity therapy. This review will focus on recent progress in the design and development of antiobesity agents which function primarily at peripheral sites. These include: 1) agents which suppress appetite primarily through interactions at peripheral sites such as the gastrointestinal tract or liver; 2) drugs which decrease the intestinal absorption of dietary carbohydrate or fat; 3) agents which alter lipid metabolism by decreasing lipid synthesis and/or enhancing lipid mobilization; 4) drugs which enhance the oxidation of lipid and/or carbohydrates and increase the ratio of heat to energy production and 5) agents which normalize hormone levels.

Several recent reviews have described novel pharmacological approaches which function by modulating peripheral sites of appetite regulation or energy metabolism (Sullivan and coworkers, 1980; Sullivan and Comai, 1978; Sullivan and Triscari, 1978).

ANORECTIC AGENTS WHICH FUNCTION AT PERIPHERAL SITES

A number of endogenous substances and experimental drugs have been described recently which appear to suppress food intake by modulating peripheral sites involved in appetite regulation. The gastrointestinal hormone cholecystokinin (CCK) is a putative short-term satiety hormone whose satiety inducing effect has been shown in rats (Gibbs and coworkers, 1973), Rhesus monkeys (Falasco and coworkers, 1979) and humans (Sturdevant and Goetz, 1976; Kissileff and coworkers, 1979; Stacher and coworkers, 1979). CCK produced a dose-dependent decrease in liquid diet intake in Rhesus monkeys implanted with open gastric cannulas; behavioral satiety was also observed (Figure 1). CCK appears to act at peripheral rather than CNS sites (Nemeroff and coworkers, 1978), although CCK-like immunoreactivity has been identified in human (Rehfeld and Kruse-Larsen, 1978) and rat brains (Innis and coworkers, 1979; Schneider and coworkers, 1979). Bombesin (BBS), another gastrointestinal peptide hormone, suppressed food but not water intake in rats and may also be a satiety signal (Gibbs and coworkers, 1979). These studies on CCK and BBS suggest that peptides have a role in satiety and encourage the search for peptide analogs as anorectic agents.

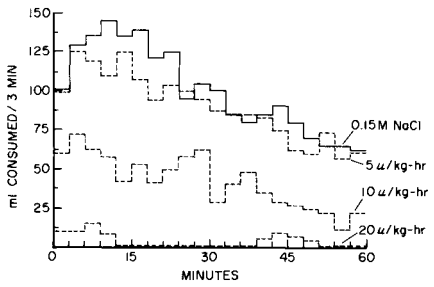

Fig. 1. Effect of slow intravenous infusions of CCK on sham feeding of liquid food in Rhesus monkeys. (Reprinted with permission from Falasco, J. D. and coworkers, 1979: Physiology & Behavior 23, 887-890).

The elevation of body glycerol concentration by multiple daily injections of glycerol led to hypophagia and body weight loss in rats; this was followed by normal food intake and a normal rate of body weight increase (Wirtshafter and Davis, 1977). A reduction in food consumption was also demonstrated in rats after the injection of adenosine, and to a lesser extent inosine (Capogrossi and coworkers, 1979). Since glycerol and adenosine are released from adipose tissue, these compounds may play a role in providing feedback signals between the adipose organ and the hypothalamic centers of food intake regulation.

That peripheral cholinergic mechanisms are involved in feeding regulation was suggested by acute experiments in rats in which atropine methyl nitrate inhibited the sham feeding of a liquid diet, but not drinking, and elicited behavioral satiety (Lorenz and coworkers, 1978). Scopolamine methyl nitrate suppressed food intake and caused weight loss in ventromedial hypothalamic-lesioned obese rats, although food intake returned to pretreatment levels and a significant amount of obesity was maintained (Carpenter and coworkers, 1979).

Recent studies suggest that prostaglandins may also play a role in appetite regulation. The intraventricular administration of prostaglandin $F_{2\alpha}$ reduced food consumption in food deprived and in satiated rats, suggesting that the hypophagic effect of this compound occurred at both peripheral and central sites (Doggett and Jawaharlal, 1977a). The involvement of prostaglandin generating systems was also suggested since the oral and intraperitoneal administration of the prostaglandin precursors arachidonic, linolenic and linoleic acids also inhibited food intake in hungry rats (Doggett and Jawaharlal, 1977b). Prior treatment with indomethacin and paracetamol reversed the anorexia and the behavioral satiety induced by the three fatty acids, but had no effect on prostaglandin $F_{2\alpha}$-induced suppression of food intake.

(−)-Hydroxycitrate and (±)-trans-epoxyaconitic acid are experimental compounds that produced anorexia and promoted weight loss apparently by modifying peripheral, rather than central mechanisms regulating appetite (Sullivan and coworkers, 1977a; Sullivan and Triscari, 1976). No CNS stimulation was observed with these agents. The appetite suppressing effect of (−)-hydroxycitrate was thought to result from an alteration in peripheral metabolite flux since fatty acid synthesis was inhibited (Sullivan and coworkers, 1977a) and, concomitantly, glycogen synthesis and levels were increased, even under pair-feeding conditions (Sullivan and Triscari, 1976). The diversion of hepatic metabolite flux from fatty acid synthesis into glycogen synthesis was suggested to affect hepatic glucoreceptors which are thought to monitor energy availability in the liver. This information may then be processed centrally to determine changes in feeding behavior. (−)-Hydroxycitrate significantly reduced food intake and body weight due to a selective reduction in body fat in several obese rodent models (Sullivan and Triscari, 1977). No alterations in fatty acid or glycogen synthesis were produced by (±)-trans-epoxyaconitate. Its anorectic activity appeared to be related to a selective reduction in the rate of gastric emptying (Sullivan and Triscari, 1978).

A new anorectic agent, (−)-threo-chlorocitric acid, was also devoid of CNS stimulatory activity, and treated rats were resistant to the development of tolerance. The oral administration of (−)-threo-chlorocitric acid reduced food intake in lean and obese rats and in dogs; anorectic potency was approximately 40-fold greater in dogs than in rats. A dose-dependent decrease in food intake and body weight gain was observed in rats meal-fed a high carbohydrate diet (Figure 2). In long-term studies in rats the reduction of food intake following treatment resulted in a significant loss of body weight which could be accounted for by a decrease in total body lipid; body protein levels were unchanged. Unlike (−)-hydroxycitrate which is structurally similar, (−)-threo-chlorocitric

acid had no effect on hepatic fatty acid synthesis. However, (−)-threo-chlorocitric acid like (±)-trans-epoxyaconitic acid suppressed significantly gastric emptying suggesting that a site of action is the upper gastrointestinal tract (Triscari and Sullivan, 1980).

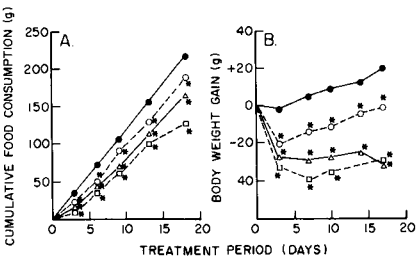

Fig. 2. Effect of (−)-threo-chlorocitric acid on food consumption and body weight gain in female Sprague-Dawley rats. Six to 10 rats per group, weighing 195 to 210 grams, were meal-fed a single chow meal daily from 8 to 11 a.m. for 7 days. Rats were treated daily with (−)-threo-chlorocitric acid at doses of 57 (0), 114 (Δ) and 227 (□) mg/kg, given orally 30 min before the meal for 17 days.

PERIPHERAL EFFECTS OF ANORECTIC AGENTS WHICH FUNCTION AT BRAIN SITES

Phenethylamines and other anorectic agents have been implicated to affect peripherally lipid and carbohydrate metabolism. Both fenfluramine (Butterfield and Wichelow, 1968) and mazindol (Turner, 1978) increased glucose uptake by human skeletal muscle but apparently through different mechanisms. A congener of fenfluramine, benfluramate, has been reported also to enhance glucose utilization in obese patients (Asmal and coworkers, 1977). Recently, a review of the peripheral actions of fenfluramine has appeared (Turner, 1979).

Fenfluramine has been reported to reduce in vitro synthesis of triglycerides in human adipose tissue (Ashwell, 1974) and rat liver (Brindley and Bowley, 1975a) and inhibit rat liver phosphatidate phosphohydrolase (Brindley and Bowley, 1975b). When administered to rats after a meal, to avoid its anorectic properties, fenfluramine reduced in vivo hepatic fatty acid synthesis (Comai and coworkers, 1978). These authors also reported a similar effect for amphetamine. Several reports have indicated that fenfluramine decreased intestinal fat absorption (Garattini and coworkers, 1975; Comai and coworkers, 1978; Curtis-Prior and coworkers, 1980). Comai and coworkers (1978) reported that the inhibition of lipid absorption by fenfluramine was dose-dependent and probably related to inhibition of pancreatic lipase. The significance of the peripheral metabolic effects of fenfluramine and other phenethylamines must be considered questionable since these effects occur at doses 10 to 100-fold greater than the human clinical dose, and careful studies have failed to show significant weight loss in addition to that produced by food reduction alone (Hipkin and Davis, 1976). Thus, in spite of the accumulating evidence of peripheral metabolic effects, the mechanism of action of currently marketed anorectic agents is their central effect on food consumption.

AGENTS WHICH REDUCE THE AVAILABILITY OF NUTRIENTS THROUGH MODULATION OF INTESTINAL ABSORPTION

Limiting the availability of nutrients to the body by altered intestinal absorption appears to be a reasonable approach to obesity therapy. The success of the intestinal bypass surgery in reducing both food consumption and nutrient absorption has given impetus to the search for orally active agents which either reduce

selectively the absorption of carbohydrate or lipid or reduce nonselectively the absorption of all nutrients.

In addition to obesity therapy, limiting carbohydrate digestion and absorption may be useful in managing other carbohydrate-dependent diseases such as maturity onset diabetes, a condition of increasing prevalence in our western culture. Retardation of carbohydrate absorption may be achieved by inhibitors of α-glucosidase (maltase), α-amylase and sucrase. By decreasing the activity of one or all of these enzymes, a decrease in the rate of absorption of glucose and an attenuation of the post-prandial hyperglycemic and insulin response would be expected. This diminished response should lead to decreased fat synthesis from carbohydrate. Initial efforts to attenuate carbohydrate digestion focused on inhibitors of α-amylase to decrease starch digestion. Inhibitors of α-amylase isolated from microbial origins, Bay d 7791 and Bay e 4609, decreased the intestinal digestion of starch and diminished the hyperglycemia and serum insulin responses to starch ingestion in healthy volunteers and diabetic patients (Puls and Keup, 1975; Frerichs and coworkers, 1974). These compounds were devoid of sucrase inhibiting activity and ineffective in tests in which sucrose was administered. Recently, Bay g 5421 was reported to possess both sucrase and maltase inhibiting properties as well as α-amylase inhibition (Schmidt and coworkers, 1977). In the genetically obese Zucker rat, Bay g 5421 dose-dependently reduced body weight gain and body and blood lipids (Figure 3) (Puls and coworkers, 1977). In lean and obese human volunteers, Bay g 5421 inhibited post-prandial increases in blood glucose, serum insulin and serum triglycerides (Hillebrand and coworkers, 1979). In a study of insulin-treated and sulphonylurea-treated diabetics Bay g 5421 reduced mean and maximal postprandial blood glucose levels in both groups (Sachse and Willms, 1979). Long-term studies on the effects of Bay g 5421 on body weight of diabetic and nondiabetic patients are awaited.

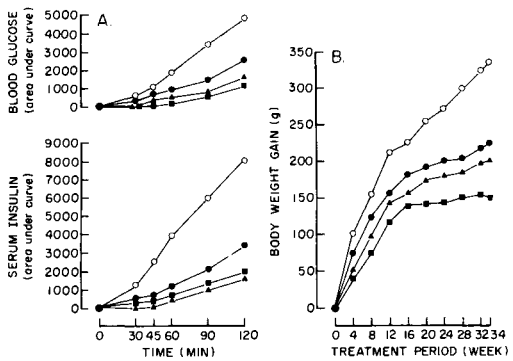

Fig. 3. Effect of Bay g 5421 on blood glucose and serum insulin in humans (Panel A): control (0), 75 mg (●), 150 mg (▲), 300 mg (■); and on body weight gain in obese Zucker rats (Panel B): control (0), 20 (●), 40 (▲) and 80 (■) mg/100 g food. (Reprinted with permission from Puls, W. and coworkers, 1977: Naturwissenshaften 64, 536-537).

Cycloleucine (1-amino-1-cyclopentane carboxylic acid) reduced significantly the efficiency of food utilization and body weight gain in rats without effect on food consumption (Aranda and coworkers, 1979). This antiobesity effect of cycloleucine was attributed to a specific reduction in pancreatic α-amylase activity; activities of sucrase, maltase, lactase and pancreatic lipase were not affected significantly.

The decreased body weight gain observed in rats treated with 2,2-dimethyl-1-(4-methyl phenyl)-1-propanone (SaH 50-283) appeared to be due to a decrease in

maltase activity in the intestinal brush border (Ho and Aranda, 1979). Food consumption was decreased at high doses of SaH 50-283, but food efficiency was reduced significantly at all doses tested, suggesting that starch digestion and absorption was inhibited.

A variety of chemically distinct compounds have been reported which reduce selectively the absorption of dietary lipid: an antifungal agent, nonabsorbable dietary fats, surface active agents and bile salt sequestering agents. Nonabsorbable dietary fat replacements and surface active agents are attractive as antiobesity agents since they might be expected to interrupt the function of pancreatic lipase, the enzyme responsible for the hydrolysis of emulsified dietary fat.

Decreased fat absorption was demonstrated in obese subjects treated with neomycin during a short-term study (Faloon and coworkers, 1966). The steatorrhea corresponding to 20% to 50% of consumed dietary fat was due apparently to precipitation of micellar lipid in the small intestine (Thompson and coworkers, 1971). Neomycin causes alterations in the intestinal mucosa at the dosage required for steatorrhea (6 g/day) and therefore its use in long-term studies is prohibitive (Dobbins and coworkers, 1968). Cholestyramine, a bile salt sequestering agent, has been used also to produce steatorrhea in humans. However, the degree of malabsorption has been too small to produce weight loss (Bray, 1976).

A specific approach to decreased fat absorption through inhibition of pancreatic lipase has been reported (Comai and Sullivan, 1980). Pluronic L-101, a lipophilic nonionic surfactant copolymer of polyoxyethylene (90%) and polyoxypropylene (10%), is a potent in vitro inhibitor of human pancreatic lipase. When administered to meal-fed Charles River rats as a 1% or 3% dietary admix Pluronic L-101 produced dose-dependent decreases in dietary fat absorption and body weight gain (Figure 4) without effects on food consumption. Pluronic F-68, a hydrophilic polymer was inactive. The decreased body weight gain produced by Pluronic L-101 was associated with a decreased percentage of carcass fat; percentage of carcass protein was unchanged. During administration as a 3% dietary admix to obese Zucker rats, Pluronic L-101 also produced a significant decrease in body weight gain. No effects on liver weight or on serum glucose, triglycerides or cholesterol levels were observed in either lean Charles River rats or genetically obese Zucker rats.

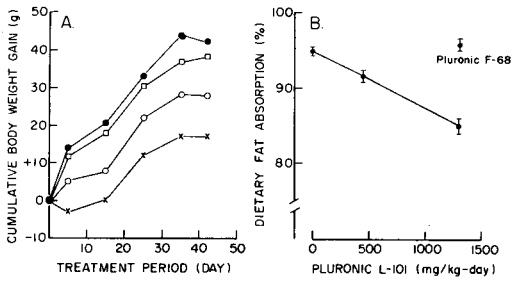

Fig. 4. Effect of Pluronic L-101 on body weight gain (Panel A) and dietary fat absorption (Panel B) in lean rats. Panel A, body weight gain: control (●); Pluronic L-101, 420 mg/kg (0); Pluronic L-101, 1272 mg/kg (X); Pluronic F-68, 1320 mg/kg (□).

Replacing conventional dietary fat with synthetic or nonabsorbable fats has been suggested to have application in obesity management. When conventional dietary

fat was replaced with medium chain triglycerides in the diet of rats, there was a decrease in body weight associated with a significant decrease in epididymal and perirenal fat pad weights after eight weeks of treatment (Lavau and Hashim, 1978). The reductive effect of medium chain triglycerides on body fat was attributed to a lack of deposition of this type of fat in adipose tissue, extensive oxidation by liver and a decrease in liver lipogenic activity. Recently, this effect was not confirmed in the genetically obese Zucker rat (Bach and coworkers, 1980). Sucrose polyester (SPE) a noncaloric, nonabsorbable fat substitute has been evaluated in rats (Mattson and coworkers, 1976) and in man (Fallat and coworkers, 1976). Recent work has indicated that SPE has greater promise as a cholesterol lowering agent than as an antiobesity drug (Glueck and coworkers, 1979; Crouse and Grundy, 1979).

Nonselective prevention of nutrient absorption has been reported with perfluorooctyl bromide (PFB) (Hussain and coworkers, 1977). PFB is a chemically and biologically inert high molecular weight fluorocarbon which coats the stomach and intestine. When administered to rats during a 3 hour meal, PFB prevented body weight gain during a 13 day study. There has been no confirmation of this study.

INHIBITORS OF LIPID SYNTHESIS

Several compounds inhibit key enzymes in lipid synthesis and concomitantly suppress body weight gain. (−)-Hydroxycitrate is a potent competitive inhibitor of ATP citrate lyase (Watson and coworkers, 1969), the extramitochondrial enzyme which supplies 2-carbon units for fatty acid and cholesterol synthesis. The inhibition of rat hepatic fatty acid synthesis in vivo by (−)-hydroxycitrate was observed after a single administration by intraperitoneal injection or oral gavage (Lowenstein, 1971; Sullivan and coworkers, 1972), or after long-term treatment by oral gavage or as a dietary admixture (Sullivan and coworkers, 1974a; Chee and coworkers, 1977). The inhibition of hepatic fatty acid synthesis following a single oral administration of (−)-hydroxycitrate was dose-dependent (Sullivan and coworkers, 1974a) and was sustained for 6 hr after treatment (Figure 5A). The (−)-hydroxycitrate-induced suppression of hepatic fatty acid synthesis resulted in a reduction of circulating triglycerides in normal rats,

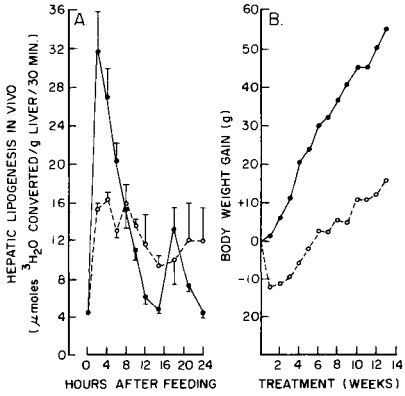

Fig. 5. Effect of (−)-hydroxycitrate on hepatic lipogenesis and body weight gain in Sprague-Dawley rats. Panel A, Lipogenesis: Two to three month old rats (10 per group) were meal-fed a 70% glucose diet for 6 days. Rats were then given a single oral dose of either saline (●) or 2.63 mmole/kg (−)-hydroxycitrate (○) immediately before receiving 8.7 g of food. Panel B, Body weight gain: Ten month old rats were fed ad libitum either a 70% glucose diet (●) or the glucose diet containing 52.6 mmole/kg diet (−)-hydroxycitrate (○).

hypertriglyceridemic obese Zucker rats and rats made hypertriglyceridemic by triton injection or the addition of fructose to their drinking water (Sullivan and coworkers, 1977a & b). In addition to its hypotriglyceridemic action, the long-term administration of (−)-hydroxycitrate as a dietary admix resulted in a significant suppression of body weight gain (Figure 5B), and total carcass lipid in mature lean rats, obese goldthioglucose treated CBA/J mice and in lean and obese Zucker rats (Sullivan and coworkers, 1974b; Sullivan and Triscari, 1977; Greenwood and coworkers, in press).

Dehydroepiandrosterone (DHA) is an inhibitor of glucose-6-phosphate dehydrogenase (McKerns and Kaleita, 1960), an important enzyme in the production of reducing equivalents for fatty acid synthesis. Inhibition of fatty acid synthesis *in vitro* was demonstrated with DHA and shown to be reversible with the addition of NADPH (Ziboh and coworkers, 1970). DHA inhibited hepatic and adipose tissue fatty acid synthesis following long-term administration to A^{vy}/a mice (Yen and coworkers, 1977). The suppression of lipogenesis was accompanied by decreased body weight gain and a reduction in total body fat (Figure 6). Appetite was not suppressed by DHA (Yen and coworkers, 1977). Another glucose-6-phosphate dehydrogenase inhibitor, 5α-androston-17-one, suppressed lipid accumulation and decreased body weight gain in A^{vy}/a mice, although an effect on lipid synthesis was not observed (Yen and coworkers, 1978).

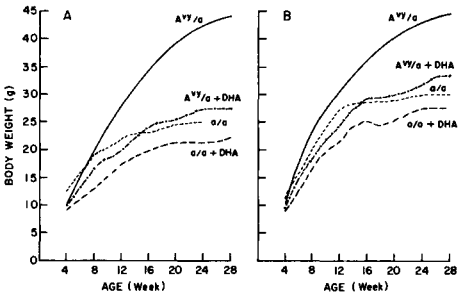

Fig. 6. Effect of dehydroepiandrosterone (DHA, 500 mg/kg p.o. three times weekly) on body weight gain in female (Panel A) and male (Panel B) A^{vy}/a and a/a mice. (Reprinted with permission from Yen, T. T. and coworkers, 1977: Lipids, 12, 409-413).

Nafenopin is a competitive inhibitor of acetyl CoA carboxylase (Maragoudakis, 1969), the rate-limiting enzyme of fatty acid synthesis. The administration of nafenopin as a dietary admixture (Best and Duncan, 1970; Leighton and coworkers, 1975; Arch and coworkers, 1980) or by oral gavage (Levine, 1974; Levine and coworkers, 1975) reduced body weight gain in rats and mice. The decreased body weight in mice resulted from the decreased accumulation of carcass lipid (Arch and coworkers, 1980). Nafenopin also increased peroxisomal proliferation (Reddy and coworkers, 1980), which may result in an increased oxidation of fatty acids (Lazarow and deDuve, 1976; Lazarow, 1978) and in turn, contribute to its antiobesity effect. Unfortunately, peroxisomal proliferation has also been correlated recently with hepatic carcinogenesis (Reddy and coworkers, 1980).

Fenfluramine, norfenfluramine, and benfluorex inhibit phosphatidate phosphohydrolase (Sturton and Brindley, 1977; Brindley and Bowley, 1975a; Brindley and coworkers, 1975), a key enzyme in triglyceride synthesis. Fenfluramine also inhibited fatty acid and cholesterol synthesis in isolated rat hepatocytes and *in vivo* (Comai and coworkers, 1978). However, as mentioned earlier in this review, the effect of fenfluramine and other fenfluramine analogs on reducing weight gain in obese patients (Dent, 1978; Noble, 1979; Vague and Tramini, 1979; Asmal and

coworkers, 1977) is probably due to their anorexigenic activity (for a review see Sullivan and coworkers, 1980; Wales, 1980).

Obese (ob/ob) mice treated with oxytetracyline demonstrated reduced levels of adipose tissue and hepatic total lipid synthesis in vivo (Begin-Heick and Heick, 1976). Hepatic glycogen synthesis (Begin-Heick and Heick, 1976) and content (Dubuc and coworkers, 1978) were increased by oxytetracycline treatment. Oxytetracycline decreased body weight in mature but not young ob/ob mice; these reductions were due to reduced carcass lipid levels (Dubuc and coworkers, 1978). Oxytetracycline suppressed serum glucose and insulin levels in mature ob/ob treated mice compared to diet-restricted controls (Dubuc and Willis, 1978). The decreased circulating insulin levels are thought to be the result of the regranulation of pancreatic islet cells caused by oxytetracycline treatment (Begin-Heick and coworkers, 1979).

A new class of compounds reduced body weight gain by decreasing carcass fat content (Cox and coworkers, 1978). The most active compounds, stilbene analogs, suppressed food intake, circulating insulin levels, and fatty acid synthesis from lactate. It was postulated that the antiobesity activity of these compounds was due to the inhibition of fatty acid synthesis, since compounds without this activity had no effect on body weight (Cox and coworkers, 1978).

AGENTS WHICH DECREASE THE EFFICIENCY OF LIPID AND/OR CARBOHYDRATE OXIDATION

Antiobesity activity should be achieved with agents that increase oxygen consumption and, therefore, lipid utilization. Thyroid hormones induce a number of changes in obese subjects which tend to enhance weight loss, the major effect is stimulation of O_2 consumption with increased utilization of endogenous fuel (Bray, 1977; Gonzales-Barronco and coworkers, 1975; Hofmann and coworkers, 1975). Although approximately three fourths of the calories expended as a result of treatment with T_3 resulted from catabolism of fat (Bray and coworkers, 1973), a majority of early weight loss resulted from catabolism of protein. Although recent studies suggested that a higher protein content in the diet could overcome this catabolic effect (Lamki and coworkers, 1973), a more potentially dangerous side effect is the modification of chronotropic and inotropic properties of cardiac muscle by thyroid hormones (Freedberg and Hamolsky, 1974). The mechanism of these effects is not known.

Dinitrophenol, an uncoupler of oxidative phosphorylation, reduced body weight in obese subjects (Tainter and coworkers, 1935), but its toxicity precludes its use as an antiobesity agent (Simons, 1953). Aspirin which is also an uncoupler of oxidative phosphorylation and which increases oxygen consumption and heat production (Smith and Dawkins, 1971; Bray, 1976) has limited application for antiobesity therapy because of its many side effects when administered at an effective antiobesity dose (Collier, 1969).

Agents which might increase lipid oxidation may have some utility in antiobesity therapy. A role for brown adipose tissue (BAT) has been postulated in obesity because of its function in β-oxidation and thermogenesis (Himms-Hagen, 1979; Rothwell and Stock, 1979). BAT is an important tissue in the maintenance of normal body temperature and its activity is increased during cold-stress induced heat production in nonshivering thermogenesis. Both the ob/ob mouse and the Zucker fatty (fa/fa) rat have been shown to be incapable of maintaining their body temperatures during cold stress (Trayhurn and coworkers, 1976; Levin and coworkers, 1980a). A possible reason for this abnormality in the ob/ob mouse has been suggested as a defect in BAT metabolism (Himms-Hagen and Desautels, 1978).

An active antiobesity agent, oxytetracycline, increased lipoprotein lipase activity in muscle and in BAT in cold-acclimated normal rats (Begin-Heick and Heick, 1977). The pharmacological intervention in BAT metabolism may prove useful in the treatment of obesity.

AGENTS WHICH ENHANCE LIPID MOBILIZATION

Agents which enhance lipid mobilization may have utility in weight control and may prevent maintenance of the obese state. Naturally occuring stimulators of lipolysis, the catecholamines, especially epinephrine and norepinephrine are potent in vivo inducers of fat release from adipose tissue (Steinberg, 1972; Fain, 1973). The genetically obese Zucker rat has been shown to possess disorders in both central and peripheral catecholamine metabolism (Levin and Sullivan, 1979 a & b; Levin and coworkers, 1980a) and obese humans do not respond to norepinephrine with an increased metabolic rate (Jung and coworkers, 1979). These factors suggest that a defective autonomic function may be in part responsible for the development and/or maintenance of obesity. However, catecholamines are potent sympathomimetic drugs and powerful cardiac stimulants. Their short duration of action and undesirable side effects such as anxiety, increased blood pressure and palpitations, limits their use as antiobesity agents.

Defects in catecholamine metabolism and thermogenesis in obesity has led to a recent proposal that a malfunctioning of brown adipose tissue (BAT) may be an important factor in obesity (Himms-Hagen, 1979). Unlike white adipose tissue, BAT from rats has been shown to possess α-receptors (Itaya, 1978) and ephedrine treatment of obese mice resulted in stimulation of BAT and decreased body fat (Miller, 1979). In further support of these observations, phenoxybenzamine, an α-blocking agent which irreversibly blocks α-adrenergic receptors was shown to enhance lipolysis in vivo in genetically obese Zucker rats during chronic administration (Levin and coworkers, 1980b). Body weight of the obese rats was reduced significantly without effects on food consumption. Phenoxybenzamine increased the outflow and turnover of norepinephrine causing increased sympathetic activity (Langley and Weiner, 1978) and was concentrated in BAT (Masuoka and coworkers, 1967). Increased sympathetic activity in adipose tissue resulting in enhanced lipolysis has been observed also in myo-inositol deficiency (Hayashi and coworkers, 1978).

Inhibitors of prostaglandin biosynthesis have been proposed as antiobesity agents since prostaglandins have been described as antilipolytic agents (Böhle and coworkers, 1966). It has been reported that indomethacin, a prostaglandin synthesis inhibitor, decreased body weight gain in obese Zucker rats (Curtis-Prior, 1975). However, in studies of patients on long-term, high dose aspirin therapy, which would be expected to inhibit prostaglandin synthesis, no changes in body weight were observed (Kather and coworkers, 1978).

Currently there are no antiobesity drugs marketed which have as their specific mechanism of action the stimulation of fatty acid release from adipose stores. This is an area which represents considerable potential.

AGENTS WHICH NORMALIZE HORMONE LEVELS

Obesity in animals and man is characterized by several hormone imbalances the most striking of which is hyperinsulinemia (Bray, 1976). Other abnormalities include diminished growth hormone secretory responsiveness, lowered glucagon secretory responsiveness and elevated cortisol secretion and turnover rates (for

review see Bray, 1976). Pharmacological approaches to normalize these hormonal imbalances merit attention.

Oxytetracycline has been discussed previously (see inhibitors of lipid synthesis) as suppressing insulin levels and body weight in ob/ob mice (Dubuc and Willis, 1978), possibly by inhibiting insulin release from pancreatic islet cells (Begin-Heick and coworkers, 1979).

Although somatostatin inhibits insulin, glucagon and growth hormone release (for review see Gomez-Pan and Hall, 1977), an analog which inhibits selectively insulin secretion may prove useful as an antiobesity agent. Attempts to produce a long acting analog have failed thus far. Preferential insulin inhibition was reported using Des-(Ala^1-Gly^2)-somatostatin, Des-(Ala^1-Gly^2-Asn^5)-somatostatin and Des-Asn^5-D-Trp^8-somatostatin (Brown and coworkers, 1976; Efendic and coworkers, 1975). In man both the linear (reduced) and cyclic (oxidized) form of somatostatin appeared to be more potent in suppressing insulin release as compared to glucagon release (Leblanc and Yen, 1975).

Pancreatic polypeptide is a recently identified hormone produced by pancreatic endocrine cells. The intraperitoneal administration of bovine pancreatic peptide reduced food intake and body weight gain in hyperphagic ob/ob mice (Malaisse-Lagae and coworkers, 1977). Avian pancreatic polypeptide and bovine pancreatic polypeptide given intraperitoneally returned the hyperinsulinemia, hyperglycemia and weight gain of New Zealand obese mice to normal (Gates and Lazarus, 1977). This normalization was also produced by the intraperitoneal implantation of islet cells from white mice to New Zealand obese mice; the implanted islets secreted mouse pancreatic polypeptide.

Weight loss was reported in aged Parkinsonian patients treated with l-dopa for many months (Vardi and coworkers, 1976). L-dopa stimulated growth hormone secretion in normal subjects although obese subjects were unresponsive (Gragnoli and coworkers, 1977; D'Alessandro and coworkers, 1977). However, a combination of l-dopa plus propranalol stimulated markedly plasma growth hormone levels in obese subjects (Barbarino and coworkers, 1978). During dietary restriction in obese patients, l-dopa increased energy expenditure and produced a greater reduction of body weight than diet alone (Shetty and coworkers, 1979). Although an anorectic effect of l-dopa was reported, recent evidence in genetically obese rats demonstrated that the sustained body weight decline following l-dopa could not be accounted for by reduced food consumption alone (Hemmes and coworkers, 1979).

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Agents Acting on Mucociliary System

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ABSTRACT

The pharmacodynamic mechanisms by which a substance can be an active agent on the mucociliary system are very different ones: stimulation of the ciliary activity, increase of the bronchial secretion, modification of its biochemical composition, normalization of the characteristics of the mucus, etc. Therefore, it is necessary to establish a series of definitions of the different pharmacological groups included within the group of the mucociliary agents. The most representative substances of each one of the pharmacological groups and their chemical structures are commented. Finally, a new technique is described, it allows to carry out a complete study of the effects of the mucociliary agents in non-anesthetized animals over long periods of time, with collection of abundant samples of bronchial mucus secreted under practically physiological conditions; from these samples, the analysis of their physicochemical parameters and of their biochemical composition can be undertaken.

KEYWORDS

Mucociliary system; expectorants; mucolytics; mucoregulators; bronchosecretors; ciliostimulants; mucus collection.

INTRODUCTION

The figure shows the known diagram of the tracheobronchial coating, where the mucus-secretion system and its transport system coexist. This diagram gives us an idea of the complexity of the pharmacological group, since the substances which may modify the bronchial secretion or its transport can act in very different ways and spots which have been marked with arrows and numbers in the picture (Fig. 1).

The secretion takes place: on one hand, in epithelial goblet cells (1), which secrete directly onto the bronchial light and on the other hand, in true glands which penetrate up to the submucosa. The latter are themselves formed by cells of mucous type (in the gland neck) (2), and of serous type (in the body and bottom of the gland) (3), their secretion being collected in the duct, so that it flows outwards. Drugs can act on quantity (4), and quality (5), of secretion, and even on the number of goblet and ciliated cells (6).

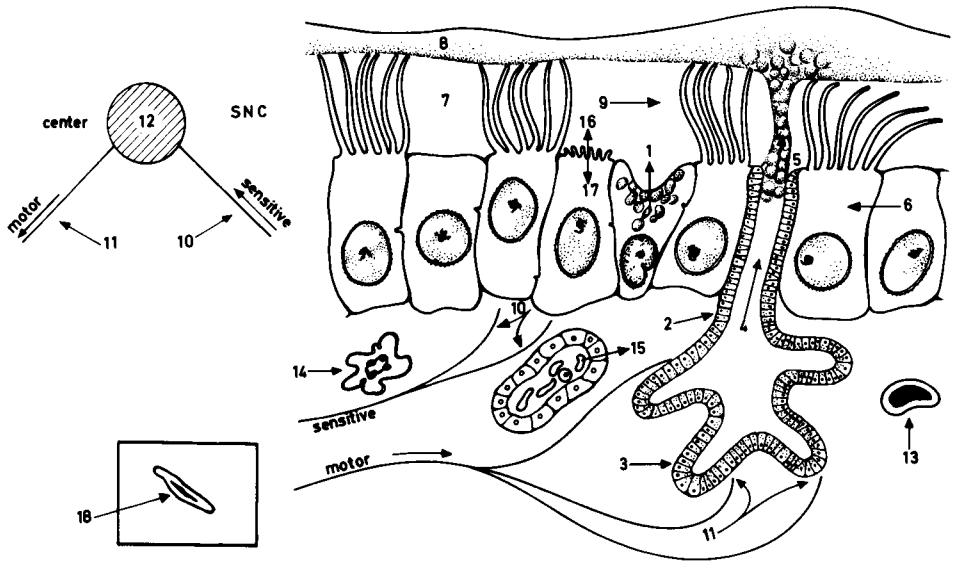

Fig. 1.- Mucociliary system. Spots of drug action. See the text.

The secreted mucus forms two well differenced layers: the lower one, a sol (7) which rests upon the epithelial cells, and the top layer, of greater viscosity and elasticity, as a gel (8), which sticks to its surface the particles, foreign bodies, bacteria, etc. which pollute the breathing air that are in its turn transferred towards the pharynx, in order to be eliminated by deglutition.

The transport system is formed by ciliated epithelial cells. Their cilia beat through the sol-phase (9), the tips touching the supraciliary gel-phase. This push of the cilia on the lower face of the mucus blanket attains the transport in the form of an endless belt thanks to the syncronization of the ciliary movements and to the fact that they are pushing in one only direction, as the cilia retract when returning to starting point.

There is a sensitive innervation (10) capable of inducing a response to several stimulus - mechanical, thermic, electric, chemical - in very different fields - respiratory and digestive - and another motor one (11), which acts upon the secretory glands and probably also upon the goblet cells, although this has only been verified in some animal species; this innervation is p-sympathetic, but in some species the presence of sympathetic fibres has also been shown. Obviously, these sensitive and motor ways have links with higher nervous centers (12).

Besides this, some of the components of the mucus have their source in the migratory cells, the lymphocytes (13) and polymorphonuclears (14) that circulate through this area.

Furthermore, there is a dynamic of liquids, salts and other substances at various levels, for instance: the transudation of proteins of plasmatic origin at capillary level (15); the hydration of the native mucin trhough the cells of the collector duct of the glands; the ion pump (16) between epithelial cells and the lower layer of the mucus, and the partial reabsorption of the latter (17).

It must also be kept in mind that the alveolar surfactant, segregated by the pneumocytes type II (18) is added to the liquid of the respiratory tract, granting it special characteristics; thence, the substances stimulating its production will influence the transport of mucus.

Due to the high number of eventual spots of action and to the diverse mechanisms of the drugs which act upon the respiratory mucus and its transport, it is necessary to define a series of pharmacological activities within the group of agents acting on mucociliary system.

PHARMACOLOGICAL DEFINITIONS

Traditionally the name of expectorants has been given to these drugs able to depurate the respiratory ways by means of the sputum, but this word has also been extended to other substances acting upon the quantity or quality of the respiratory mucus, without this being necessarily linked with the expectorating act.

However, since the discovery of products able to decrease the viscosity of the mucus the term "mucolytic" was coined and it was also extended to other products with different action mechanisms. Both these terms -expectorants and mucolytics- are often indistinctly used, as if they were synonyms.

Nevertheless, in order to avoid misunderstood concepts and bearing in mind the great number of action-mechanisms which are possible in this pharmacological group, I think it would be useful to give the definitions of each one of the pharmacological activities related with the mucociliary system, it being understood that one single substance can involve several of the stated actions.

In any case, the pharmacological group includes all the drugs that we can define as: agents acting on the mucociliary system.

The main effects to be outlined in the group of agents acting on the mucociliary system are the following ones:

Expectorants: all the substances that help or increase the sputum elimination.

Bronchosecretors or secretomotors: this denomination can be given both to agents stimulating the mucus secretion of the appropriate cells (goblet, serous epithelial, serous and mucous glandular cells) and to those responsible for increasing the number of goblet cells. In this case, although the speed of mucoproteins synthesis is not activated the total secretion shall increase substantially, due to the increase of the number of secretory cells.

The bronchosecretors can be of direct action, when they act directly upon the secretory cells or upon epithelial cells being transformed into these, or of indirect action, when they stimulate the sensitive nervous fibres obtaining a reflex response of increased secretion.

Ciliostimulants: agents that hasten or improve the ciliary transport system, either by a direct action upon the speed or intensity of the cilia movement, or by reestablishing their synchronization when it is altered.

Mucolytics or Secretolytics: when the viscoelasticity of the bronchial secretion has increased above the limits of transport possibility, the drugs which are able to decrease this parameter by acting upon the macromolecules forming the mucus and unfolding them into more simple ones, are the only ones that can be rightly called mucolytics.

Fluidificants: those helping the transport of the mucus blanket either by purely physical dilution mechanisms, or by diminishing the surface tension, being called in this case, obviously, tensoactives.

Ionic regulators: those substances helping the passage of the ions which have a role in the mechanism of secretion or transport: i.e., the pump of sodium and chloride ions through the microvilli in order to establish a proper osmolarity in the interciliary phase. Also those substances influencing the supply and use of calcium as an activating agent in the exocytosis process, by which the endocellular granules of the secretory cells release their products outwards.

Mucoregulators: under this term should be included the substances able to modify the biochemical composition of the secreted mucus; i.e., the quantity of the different neutral mucoproteins, or the content in sialic acids and sulfate radicals, adjusting its ratio and relative index to an optimization of the mucus functionalism.

Its mechanism can be as much by activation, as by inhibition of the enzymatic synthesis mechanisms of the different substances forming the mucoproteins and other secretion compounds.

Permeabilizants: among the substances able to modify the mucus composition, those helping the passage of the plasmatic ingredients by increasing the permeability of the capillaries or of the epithelial or glandulary cells should be underlined.

Antibronchitics: this is a far more generic denomination, that should iclude every drug able to diminish or prevent the irritation of the bronchial ways; but, since one of the main symptoms of bronchitis is the bronchial hypersecretion, this term shall be specially given to the substances that can diminish or prevent this, particularly those substances that directly or indirectly hinder -by antagonizing the effects of the aggressor products- the proliferation of goblet cells responsible for the mucus hypersecretion.

There are other pharmacological groups which can not be included among the agents acting on the mucociliary system, but which are indirectly related with it, the main ones being: tussives and antitussives, anti-infectious, anti-inflammatories and bronchodilators.

AGENTS ACTING ON MUCOCILIARY SYSTEM

Bronchosecretors:

Substances of very different kinds stimulate the bronchial secretion and they can act through diverse mechanisms. Cholinergics stimulate directly the release of muco proteins (Sturgess, 1972; Roussel, 1978), specially the sulfated ones (Parke, 1978); however, there is some discussion on if they also hasten the synthesis (Boat, 1975) or not (Sturgess, 1972). The pilocarpine acts by increasing the number of goblet cells (Reid, 1974), as also happens with the isoprenaline and related substances; the latter modify the mucus secretion towards the acid mucoproteins, this having induced cases of bronchial tamponages (Anon, 1976); it seems that this does not happen with the beta-two selective adrenergics.

Many secretor agents are merely irritative, either of indirect effect -through a gastric reflex, as the ammonium salts and the emetine- or of direct effect, among which we can mention the polluting agents (tobacco smoke, sulfurous anhydride, acids, mineral dusts...); the gaseous anesthetics (ethylic ether, halotane) and the essences (eucaliptol, gomenol, terebanthine, cedar...) among which the guaiacol and its derivatives outstand. Likewise, cold dry air induces the response of increasing the bronchial secretion (Florey, 1932), as a defense reaction.

The prostaglandins also activate the mucus secretion, the $F_{2\alpha}$ being the one that most highly increases the sputum production in the human being (Parke, 1978; López Vidriero, 1977). Having also tried the clinical application of the PG E_1 it has shown to entail an increase of the secretion and a dropping of the viscosity in the expectoration, but not higher than the one produced by the saline solution (Endres, 1978); most probably, its action is mainly due to its irritative properties.

Ciliostimulants:

Cholinergics and beta-adrenergics hasten furthermore the ciliary movements (Blair, 1969; Iravani, 1974); although this effect has been demostrated even in a certain anticholinergic, such as terolidine (Sackner, 1976; Ruffin, 1978).

On the other hand, oxygen, barbiturates (Fobes, 1976) and certain local anesthetics stop the ciliary motility (Yeager, 1970), although the lidocaine can accelerate it (Phipps, 1976).

In asthmatic processes there is a slowing down of the mucociliary transport and it has been noted that the SRS-A antagonists can prevent this; the mechanism of their action however remains unknown (Wanner, 1975).

Mucolytics:

According to the strict definition of this group, only those substances that break the links binding the macromolecules contained in the mucus should be included; since these bridges are important in order to maintain the gel-structure of the mucus, when they are broken, the mucus viscosity diminishes, and thus its transport becomes easier, particularly when there was an increased viscosity.

Reducers: Since the most stable bridges are the disulfide ones, to which the manyfold form of the macromolecules are mainly due, plus the constitution of the networks they form, substances which are able to break down the disulfide links, such as reducers, have mucolytic activity.

The ascorbic acid has been used in several reducing combinations, not only with hydrogen peroxide (Quevauviller, 1972), but mainly with sodium bicarbonate and copper sulfate (Ascoxal); "in vitro" it irreversibly breaks the mucin macromolecules, this effect being reinforced by the cupric ions, and when administered to patients in spray form, it increases the volume of the sputum, diminishing its "apparent" viscosity (Dulfano, 1969).

But, undoubtedly, the greatest part of this group, that has an action mechanism consisting in the breakage of the disulfide bridges by reduction, is formed by those substances owning a free thiol group.

The l-cysteine can be regarded as the head of the whole series, but since it is mostly unstable and irritative new compounds were investigated. Among them, one of the first which has proved to be really important is the N-acetylated which partially lessens these inconvenients, being widely used in therapeutics for this reason.

Other l-cysteine derivatives are the methyl- and ethyl-esters. All of them are active when directly administered either by spray or instillation, but N-acetylcysteine keeps enough irritative power as to show a ciliodepressor effect, also due to the thiol group (Draisin, 1979); because of this, the search for more derivatives continued both by substituents in N-group and by replacing the aminoacid. Among the first: the N-guanyl-l-cysteine, N-propyl, N-caproyl, N-benzoyl and N-mercapto derivatives (Santen Pharm, 1977). Among the second: the N-guanyl-d,l-isocysteine, N-guanyl-d,l-penicilamine, N-acetyl-d,l-penicilamine and N-acetyl-d,l-beta-mercaptoleucine (Sheffner, 1964; Rambacher, 1976). Most of them have proved "in vitro" to be as

active or more than the N-acetylcysteine, with exception of the ethyl-cysteine, that increased the mucus viscosity (Kasé, 1978), although in no significant way.

It is worth noting that the penicilamine derivatives have not proved to be particularly mucolytics, since these substances, besides their reducing power by the thiol group, have a remarkable chelating action that should have helped to open the bridges formed by the calcium ion. So, I think it would be suitable, to further study the behaviour of the penicilamine or some other of its derivatives; also noteworthy is the fact that no derivatives have been obtained from other chelant, the dimercaprol, from the same group and with two thiol-functions.

Another representative agent of the group, is the 2-thiol-propion-amide-acetic-acid or tiopronin, structural isomer of the N-acetyl-l-cysteine. Its use has been introduced in therapeutics (Chirletti, 1979; Lorenzini, 1979), since in addition to having a direct action -by inhalation it modifies the thromboelastogramme of the sputum (Finiguerra, 1979)- and unlike the former ones, is also active by parenteral way (Saba, 1978).

Several derivatives have been obtained from the tiopronin, such as the thenoyl-thio-propionyl-glycine (Chauvin, 1974) and the mercapto-aceto-benzoyl glycine (Martin, 1979); it is noteworthy that the tiopronin and its derivatives, besides the thiol group, offer the amide function, which can confer disintegrant effects.

One product whose structure differs from the former ones, oustanding from them by owning two free thiol groups is the d,l-threo-1,4-dimercapto-2,3-butanediol (dithio threitol), with a mucolytic potency "in vitro" at least ten times higher than N-acetyl-l-cysteine (Davis, 1974; Hirsch, 1969), entailing at a concentration 0.01 N the total liquefaction of the sputum after 30 minutes (Leightowler, 1971).

An inorganic product of intermediate activity between N-acetyl-l-cysteine and dithiothreitol is sodium metabisulfite, which being less S-nucleophile than the former ones, could be less toxic (McNiff, 1976).

Mercapto-ethane-sodium sulfonate (Mesna) is less irritative, has a less unpleasant odour than N-acetyl-l-cysteine (Don Barton, 1974; Buergi, 1974; Temmerman, 1971), and has proved to be at least three times more active than it (Temmerman, 1971), since to the reducing effect of the thiol group it adds that the sulfonate radical solubilizes the fractioned proteins (Steen, 1974). What is more, it has been described as able to destroy the DNA fibres found in the pathological sputum (Buergi, 1974).

Several derivatives have been obtained from this product, such as the 2-amino-pyrimidine-2-mercapto-ethane-sulfonate and the guanidinium-2-mercapto-ethane-sulfonate (Hirsch, 1969), which have a weaker activity.

Derivatives have also been described, with thiol functions free or occupied and with an amide group, from the mercapto-acetamido-benzoic acids (in different positions) and from mercapto-acetamido-sulfonic acids (Martin, 1977).

In spite of the great number of products obtained for a mucolytic purpose which is based on the activity of the thiol group, it is curious that one of the most simple ones, the mercaptoethanol, has not been kept in mind in spite of being well known that it reinforces the lythic action of the enzymes upon the cellular wall of the yeasts (Vorotilo, 1975) and is even used to destroy the gel structure in sputum analysis.

Cysteine derivatives were obtained, opened or cycled, with the thiophene group, in several forms, such as the N-(alpha-thenoyl)-l-cysteine (Chauvin, 1974), and its esters; the N-acetyl-S-thenoyl-l-cysteine and N-thenoyl-S-acetyl-l-cysteine with its

salts and esters (Blum, 1978); and several derivatives from the thienyl-thiazolidine-carboxylic acid (Di Constanzo, 1975). From all these series, some of the compounds have proved to be as active or even more than the N-acetyl-l-cysteine or than the S-carboxy-methyl-l-cysteine in "in vitro" tests or experimental bronchitis. Another derivative of the thiazolidine-carboxylic acid is the carboxy-4-thiazolidinyl-2-mercapto-ethyl acetate (letosteine), which has been introduced in clinic with clear results of volume increase of expectoration, diminishing the viscosity of the sputum (Bommartini, 1978).

The sodium salt thenoyl-homocysteine could also be mentioned (Adserá, 1976) and its thiolactone (Maiorana, 1977), although no pharmacological data of them have been provided.

Enzymes: they are also true mucolytics, since they partly destroy the molecular chains. The enzymes used as mucolytic agents can offer three kinds of different actions:

a.- The hydrolysis and separation of certain lateral residues of the chains, as in the case of the sialidases which act specifically upon the acid rests of the sugars that form the glycoproteins.

b.- The hydrolysis of the proteic structure of the mucoproteins, which may be carried out either at arginine-lysine links level, as with alpha-trypsin, or upon the carbonyl groups of the aromatic aminoacids as with chymotrypsin.

c.- Hydrolysis of non-physiological products of the sputum. In pathological conditions such as bronchitis and asthma the viscoelasticity of the mucus is increased not only by modification of its own components, but furthermore by the presence of cellular detritus, caused by the tissular destruction; here nucleotides and desoxyribonucleic acid molecules are present through breakage of the nucleus. In these cases the enzymes DNA-ase (Dornase) and the leucine-amino-peptidase behave as mucolytics, degrading such molecules (Booth, 1977).

In cases where clots may be present, streptokinase has also been used (Booth, 1977).

On one hand, proteolytic enzymes have the great advantage that they can be orally administered, but on the other hand, they offer some inconvenients: strong irritation entailing ciliodepression, and allergic reactions by reabsorption of the hydrolized proteic fractions.

However, in a study carried out in culture with rings of chicken trachea the only one drug that did not behave as a ciliotoxical was precisely the Dornase (Dudley, 1977). This should encourage us to keep on searching in Nature for enzymes that whilst being active as mucolytics are free of their peculiar local toxicity, since enzymes of this type are very often found in microorganisms (Derkanosova, 1975; Vorotilo, 1973).

The inverse effect to mucolytics is that of the substances with a tendency to create intermolecular link-bridges, as happens with the sodium tetraborate.

Ionic regulators:

Some of the intramolecular bridges have an electrovalent character between residues with charges of opposite sign or of negative sign but linked by a divalent cation, in this cases the calcium ion. Ions, on account of their concentration and the modification they cause in the electric fields, are able to open the bridges formed between residues of opposite charges.

Since the olden days certain salts have been used for their expectorant effect, the

potassium chlorate, for instance, or the association of sodium bicarbonate and copper sulfate. Their action mechanism is not yet properly defined but they probably furnish ions favouring the secretion itself or if not its optimization.

The presence of mineral salts, calcium ion and acidifying agents destroys the power of molecular association of the big molecules forming the mucus; therefore, they can have a fluidifying-mucolytic effect.

Due to the importance of the osmolarity in the sol-phase of the mucus, the substances that improved or regulated the excretion of chloride and sodium ions by the microvilli of the epithelial cells, would help to better the functioning of the depuration system.

Chelants, when kindnapping the calcium ions, open the links formed by these ions. Among the chelants, the most classical one is the EDTA, which is active "in vitro" (Jenssen, 1974).

Besides, the calcium ion is particularly important, not only because of its saline effect, but because its presence is indispensable to attain the phenomenon of the exocytosis of the granules of the secretory cells; thus, the furosemide -substance able to inhibit the calcium action- induces a decrease in the mucus secretion by preventing the release of the substances contained in the granules of the secretory cells.

Fluidificants:

These substances merely act by opening the hydrogen and hydrophobous bridges; they are, therefore, tensoactive substances; this is the point where the amides of urea-type also exert their action.

Among the amides described as mucolytics (Hirsch, 1969), only the formamide and specially the urea can be underlined. The first induces "in vitro" the partial dispersion of the gel; the effect of the urea seems far more intense (Yaeger, 1970; Khan, 1976), but only with high concentrations far higher than those available "in vivo" (Don Barton, 1974); at any rate, it is interesting to stress upon the fact that the activity seems to be rooted in the amide group. But we must add that the guanidine also shows a strong mucolytic acction "in vitro".

Concerning the tensoactives, the most used is the octyl-phenol-formaldehyde polymer (tiloxapol) of non-ionic character, whose clinical usefulness is arguable (Korsgaard, 1976), but which is usually associated with sodium bicarbonate and glycerin (Alevaire), inducing a remarkable fluidification of the mucus, and improving its transport, although an excess of fluidification can be dangerous.

Of smaller potency, inducing only an incomplete dispersion of the gel, are the anionic tensoactives such as dodecyl sulfate, and the cationic ones such as benzalkonium chloride.

The most simple of all the fluidifiers is water, which is applied in spray form, and acts by dilution of the pathological mucus, restoring the physical properties required for its normal depuration.

Dehydration, on the contrary, reduces the thickness of the blanket of mucus, which then concentrates, hindering the transport; systemic hydration however did not improve this situation (Boyd, 1968).

Mucoregulators:

This group includes a series of substances often unappropriately called mucolytics

but that in fact do not act mainly as such. They modify the chemical composition of the mucus not necessarily by increasing the secretion but only by adjusting or regulating it so that a balance among the different kinds of mucoproteins is built and the physical conditions of the mucus may be normalized for its optimal transport.

Very different substances are included in this group. On one hand, derivatives from l-cysteine, but with the thiol group occupied; they therefore do not have the mucolytic property of the aminoacid. Among these products, the most tested and widely used in clinic and, therefore, better known is the S-carboxymethyl-l-cysteine, whose main characteristics and differences with the kind of drugs above referred are: activity by oral route; pulmonary tropism, particularly by the injured cells of the bronchial epithelium (Servin, 1974, 1976); contribution of SH-groups to the glandular cells, since these groups have a tendency to vanish in the experimental bronchitis by sulfurous anhydride; increase of sialomucins by activation of the sialiltransferase or by inhibition of the sialidase (in the experimental and clinical bronchitis there is a displacement towards neutral mucins) (Rousell, 1978; Degand, 1973), or sulfate mucins (Huyen, Quevauviller, 1972); a decrease of the hyperplasia of goblet cells (Quevauviller, 1972); an increase of secretion with decrease of its viscosity (Yanaura, 1976; Muttari, 1977); and a non acceleration of the ciliary transport (Goodman, 1978). Thence, it has been wisely defined not as mucolytic, but as mucoregulator (Degand, 1973).

However, there are contradictory data, such as those sustaining that it removes or hinders the incorporation of sialic residues (Harvez, 1970), that the sialic acids content of the sputum drops (Muttari, 1977) by S-carboxy-methyl-l-cysteine, or even, that it increases the viscosity of the sputum (Puchelle, 1978).

The peculiarity of this substance consists in showing the thiol group blocked; the link -C-S-C-is stable (as in its methylic, ethylic and propylic homologous) to such an extent that it does not split in its metabolism, since it is excreted mainly unchanged, or as thiodiglycolic acid, which is an active metabolite resulting of its deamination (Servin, 1976).

As belonging to the same chemical group can be regarded the S-prenyl-l-cysteine, which experimentally behaves as a stimulant of the bronchial secretion and of the ciliary transport (Arañó, 1979; Gras, 1979; Giráldez, 1979), as much by oral as by parenteral way.

Of similar chemical structure are the N-S-diacetyl-cysteine methyl ester (mucitol) and the S-S'-methylene-bis-cysteine; this last product, of vegetal extraction, experimentally protects from the sulfurous anhydride aggression with better results than the S-carboxymethyl-l-cysteine (Causett, 1976).

Somewhat different, since it is not a cysteine derivative, but also with the link -C-S-C-is the carboxymethyl-thio-acetic acid, which also protects from the sulfurous anhydride aggression (Joullie, 1975).

From vegetal extracts used as expectorants, vasicine has been extracted and from this bromhexine was obtained being one of the substances which has a wider prestige as active agent on the respiratory mucus. This substance, also active by oral way, has been said, jointly with a slight antitussive and emetic activity, to: have a clear expectorant effect (Engelhorn, 1963); increase the sputum volume (Boyd, 1966; Paramelle, 1978); drop the viscosity of the sputum (Yaeger, 1970; Quevauviller, 1972); stimulate the secretion of the goblet cells and of the glands (Harada, 1977); hasten the clearance of radiopaques; solve the lipopolysaccharides fibres (Boyd, 1966) and stimulate the surfactant production (Cornia, 1971).

Also in this case there are different opinions, since its activity has not been

shown at all in the depuration of silicic anhydride powder (Vyskocil, 1975), and is scarce in the elimination of the respiratory fluid (Scuri, 1976; Chinese Acad. Med. Sci., 1977; Misawa, 1979); although it does not alter, or even increase, the ciliary frequency, it has been said to: it drop the speed of transport (Iravani, 1976), increase the viscosity of the mucus (Kasé, 1978), and scarcely reduce the mucoreogramme (Grassi, 1977).

Up to a certain point, the bromhexine could be regarded as a pro-drug, since one of its metabolites, the so called VIII, is in fact the active principle, which diminishes the mucus consistence and increases the ciliary activity (Iravani, 1974).

This metabolite, after being isolated (Schraven, 1967) has been synthetized, being called ambroxol; its activity is higher than the bromhexine's, as far as the stimulation of the pneumocytes type II activity is concerned, therefore, it increases the surfactant production (Curti, 1972, 1974); in other aspects, its effectiveness is also similar or higher than that of the bromhexine, including the clinical results (Ruprecht, 1978).

Among the derivatives obtained from this substance, the brovanexine has to be underlined; it increases the nasal secretion and the respiratory fluid (García Rafanell, 1976a), diminishes its viscosity (García Rafanell, 1976b) and is uniformly spread through the organism (Rimbau, 1978).

With the nucleus of the bromhexine, a methioninic derivative with the thiol group occupied was obtained (Blain, 1978), as well as derivatives from other sulfur-containing aminoacids (Blain, 1978).

Some other substances of different chemical characteristics but similar properties have also been described. One of these is the sobrerol -an alpha pinene derivative- which experimentally entails an increase of the bronchial secretion (Scuri, 1976; Kasé, 1978), but not of the alveolar surfactant (Curti, 1973); good clinical results are reported (Dalla Valle, 1970; Monzali, 1970).

There is also a substance of natural origin, extracted from a species of Rhododendron, in China, the farrerol, which besides mucoregulatory properties such as modifying the total proteins, diminishing the sialic acids and increasing the fucose, acts as a bronchosecretor directly upon the secretory cells, as a ciliostimulant, accelerating the muco-ciliary movement, and as an antipermeabilizant inhibiting the ATP-ase, this exzyme helping the cellular permeability (Chinese Acad. Med. Sci., 1977). In fact, farrerol is a derivative of eriodictyol, a flavonoid used as expectorant time ago.

Many other plants are used as expectorants in natural medicine: Caesalpinia, Primula, Angelica, Cocillana, Grindelia, Allium, Scilla, (Krishna, 1978; Misra, 1977; Shoji, 1975; Schneider, 1978).

Finally, those substances which possess an action contrary to the one we have just described, for example, they modify the composition of the mucus by confering it properties that prevent the normal transport, can, I think, be called disregulatory agents. Among these we can obviously mention all irrititative substances and those others inhibiting the protein synthesis (sodium salicylate and cycloheximide), the sugar synthesis or both, such as the ouabain (Parke, 1978; Coles, 1977).

Permeabilizants:

Apart from the vasoactive amines such as the histamine, and the kinins that help the formation of plasmatic exhudate by increasing the capillary permeability, several other substances act in a similar way. The potassium iodide, for instance, adds

to its expectorant action by gastric irritation and local effect this permeabilizant property. In this case, it can even cause oedema of the respiratory ways, particularly the gloltis (precisely one of the signs of iodism) together with an increase of nasal and bronchial secretion and onset coughing.

Furthermore, it is excreted by bronchial way, so, besides being concentrated in the spot of action, it increases the volume of the secretion by increasing the oncotic pressure (Eichle, 1958, 1964; Höbel, 1967, 1968). Even a mucolytic effect has been described in the purulent sputums, since it accelerates the activity of the proteolytic enzymes (Lieberman, 1964).

Due to its different properties (secretomotor by direct and reflex way, permeabilizant and mucolytic) plus its on-the-spot concentration, the potassium iodide could seem the ideal agent to act upon the mucociliary system, but in practice its therapeutical effectiveness is not quite clearly defined.

This has brought about the obtention of a series of iodated organic derivatives, or bromurated in other cases, the most important being the iodo-propylidene-glycerol, or iodo-dioxolane, that behaves as mucolytic, diminishing the viscosity of the sputum (Novara, 1975; Grassi, 1977), and increasing the volume of the expectoration (Cantarelli, 1979; Blasi, 1975). Being distributed more slowly, the toxicity risk is lessened.

In the substances of mixed action such as the bromhexine and its derivatives, the presence of halogen should not be forgotten.

The ATP-ase enzyme appears to be a permeability-regulator of the cellular membrane, therefore substances activating it help the exudation, whilst drugs inhibiting it, such as farrerol, decrease the proteins content of the sputum.

ASSAY METHODS

Bearing in mind the great variety of different pharmacological activities that, as we have seen, are included in the present group and the even greater number of possible action mechanisms, it is understandable that the experimental models used in the study of the biological activity of drugs acting upon the mucociliary system, can only assay very partial aspects of this.

Therefore, we can roughly divide the available methods in seven groups according to the aspect they allow to assay: 1.- Morphology. 2.- Ciliary activity and transport speed. 3.- Physico-chemical characteristics of the mucus. 4.- Secretion speed. 5.- Secretion composition. 6.- Mucus collection. 7.- Protection of the respiratory air ways.

But in addition to all these different methods that allow to study partial aspects of the mucociliary system, we suggest a new type of experimental model, by which a wide spectrum of properties and functions of the mucociliary system can be easily studied.

8.- Wide spectrum Study:
Our original technique consists in the chronic implantation of an extratracheal cannula, instead of an endotracheal one, under anesthesia but only during the surgical operation in animals that later remain fully conscious and under practically physiological conditions; the fluid of their respiratory tract (RTF) is withdrawn for several days, since the cannula bears, on the external branch, the collecting receiver, which can be easily replaced (Giráldez, 1977, 1978, 1979)(Fig. 2).

This tecnique has a lot of advantages and possibilities.

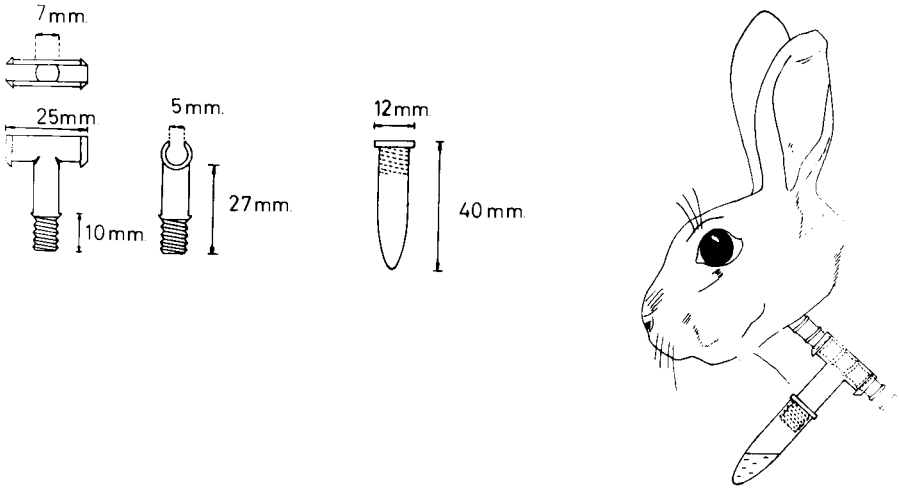

Fig. 2.- Extratracheal cannula implanted in rabbits by Giráldez-Gras method.

Advantages:

a.- The animals are not anesthetized, but remain conscious and under normal living conditions.

b.- The breathing air is conditioned by the higher respiratory ways; so, the conditions are perfectly physiological ones.

c.- Very abundant volumes of mucus sample are obtained; it is even possible to collect the total of 24 h.

d.- The experiment is carried out over long time periods, up to several days and even weeks.

e.- The same animal can undergo several successive treatments, acting as a control of itself; thus, the dispersion of results due to individual variability is reduced.

f.- The samples obtained are pure without water contamination nor dilutions involved by washings; this enables to study the physical properties with accuracy.

g.- The abundance and purity of the samples allow the exhaustive analysis of the biochemical composition of the mucus.

Possibilites:

a.- Comparative study of the composition of mucus belonging to different animal species.

b.- Study of the external conditions that influence the bronchial secretion: circadian rhythms, stationary ones, etc.

c.- Study of the variations due to different pathological conditions.

d.- Study of the influence of drugs on the secretion speed of the mucus and its composition, the normal as well as the pathological one.

e.- Connection between the visco-elastic properties and the sialic acid/fucose index.

f.- Knowledge of the drugs ruling this connection.

g.- Evaluation of the transport speed if a tracer is injected intratrachealy in a place distally settled with regard to the tracheal orifice.

h.- Study of components of secretion using labeled precursors.

i.- Study of substances of bronchial excretion and their relationship with the mucus secretion.

j.- Determination of the permeability and the transudation value in the total composition of the mucus.

k.- Evaluation of the inmunologic power of the mucus.

l.- Kinetics of the active drugs on the mucociliary system, establishing if they are or not found in the bronchial secretion.

m.- Study of the substances and active metabolites which are present in the spot of action.

RESULTS

With the formerly described technique, applied to the rabbit, the following results have been obtained, about the composition of the normal mucus (Giráldez, 1979).

TABLE I.- Physic and biochemical parameters of rabbit's RTF, collected by Giráldez-Gras method.

Parameter	N	$\bar{M} \pm$ S.E.	Unit
Weight of RTF collected	22	535.3 ± 98.7	mg/kg. animal
Total solids	9	3.6 ± 0.4	%
Density	15	0.988 ± 0.008	g/ml.
Viscosity	9	2.8 ± 0.8	c.p.s.
pH	9	7.4 ± 0.1	pH
Total proteins	11	2.9 ± 0.4	g/100 ml.
Mucoproteins	2	40.2	mg/100 ml.
-SH groups	10	132.1 ± 40.6	mg l-cysteine/g. proteins
Sialic acids	8	2.5 ± 0.2	mg/g. proteins
Fucose	7	464.3 ± 82.0	mcg/g. proteins

The following results have been obtained about the effect of several drugs in connection with the total volume of secretion (Fig. 3) (Giráldez, 1979).

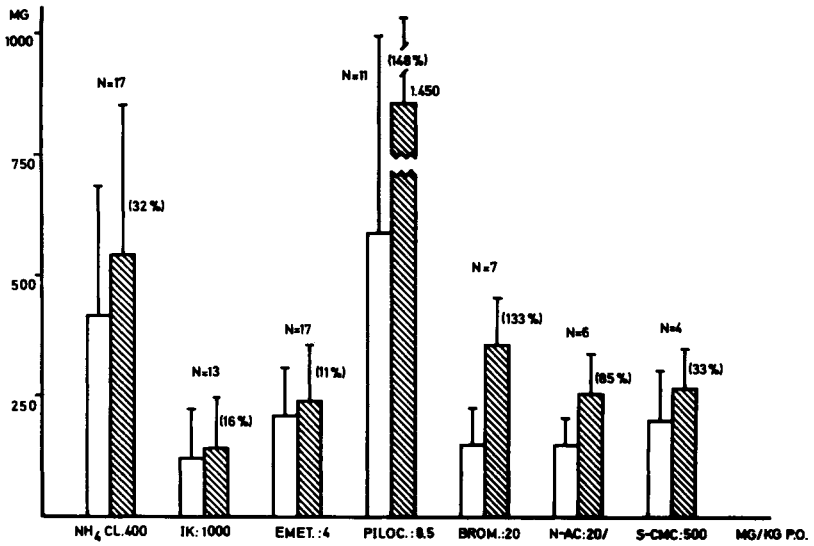

Fig. 3.- Effect of ammonium chloride, potassium iodide, emetine, bromhexine, N-acetyl-l-cysteine, and S-carboxy-methyl-l-cysteine on RTF elimination. Non anesthetized rabbits with extra-tracheal cannula implanted. Periods of 4 h.: ☐ control, ▨ after treatment.

CONCUSION

Bearing in mind the various concepts here stated, regarding the different sites of action of the mucociliary agents, the diverse pharmacological effects which optimize the system of pulmonary depuration and the different functions, chemical groups and structures found in the various kinds of drugs here commented, the researcher who wants to outline a new drug as a mucociliary agent, must succeed in guessing where to place the pieces of a very complex puzzle.

Obviously, if the purpose of the researcher would be to endow a single molecule with all te different mucociliary activities, in order to obtain the ideal drug, very likely this product should result like and hybrid, hardly to be obtained.

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Adrenergic and Anticholinergic Bronchodilators; Do They Act as Bronchospasmolytics? Perspectives in the development of anti-asthmatics

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ABSTRACT

On the basis of recent experimental evidence the classical concept of bronchospasmogens and bronchospasmolytics is challenged, switching the emphasis from bronchial smooth muscle hyperreactivity to tracheal and bronchial mucosa hypersensitivity or irritability. Against the background of the revised views some possibilities for development of new anti-asthmatic agents are discussed.

KEYWORDS

Bronchial hyperreactivity; bronchial hypersensitivity; bronchospasmogens; bronchospasmolytics; receptor up-regulation; receptor down-regulation; adrenergic bronchodilators; anticholinergic bronchodilators.

ANTI-ASTHMATIC AGENTS

In a classical sense these can be differentiated as

1. Inhibitors of mediator release:

a. Steroid derivatives such as beclomethasone which suppress immunological reactions and inhibit the release of irritant mediators from sensitized mast cells as induced by the specific antigens.
b. Cromoglycate and recently developed similarly active agents (Wasley, this volume) which act as inhibitors of irritant mediator release from mast cells (Carpenter, 1980).
c. Prostaglandins E_1 and E_2, possibly acting as inhibitors of mediator release but potentially as well acting as agents counteracting contraction of bronchial smooth muscle.

2. Bronchospasmolytics:

a. Anticholinergics, blockers of the muscarinic receptors, primarily involved in neurotransmission at the vagal nerve endings on the bronchial smooth muscle.
b. H_1-antihistamines acting as blockers of the mediator histamine in its turn considered a bronchial smooth muscle spasmogen. The fact that the anti-asthmatic potency of antihistamines is relatively poor indicates that the mediator histamine does not play a predominant role.

c. β_2-Adrenergics acting as functional antagonists of spasmogens such as acetylcholine (vagal neurotransmitter) and the mediators histamine and slow reacting substance-A (SRS-A).
d. Slow reacting substance-A antagonists? The structure of SRS-A is established (Morris and others, 1980). It appears to be derived from an epoxide of a polyunsaturated 20 carbonatoms containing fatty acid reacting with glutathion, part of which is split up then such that a 5-hydroxy-6-cystenyl-glycinyl-7,9,11,14-eicosatetraenoic acid (SRS-A) is formed. This opens the possibility to develop agents interfering with SRS-A-synthesis or antagonists blocking the receptors for this mediator. Such antagonists, too, might be considered as bronchospasmolytics.

Bronchospasmogens are substances that cause bronchial smooth muscle contraction; examples are acetylcholine (and related cholinergic compounds such as carbachol), histamine and SRS-A. They are used as inhalants to induce bronchial constriction in "provocation tests" in which the changes brought about in bronchial resistance are measured. Also other agents such as specific antigens or allergens, bronchial irritants, such as ozon, SO_2 or diluted citric acid (lowering pH), can be effectively used as provocative agents. Of particular interest is the provocation of bronchial obstruction by α-adrenergic agents, effective especially after exposure to β-adrenergic blockers (Patel, 1979); the exercise-induced bronchial obstruction, that appears to be due to an increase in ventilation and thus in evaporation of fluid at the surface of the bronchial mucosa and therewith a local cooling (with warmed up water-saturated air the exercise-induced asthma is suppressed) and the bronchial obstruction as induced by a cold, relatively large particle-size, aerosol of cooled water (cold mist, fog), where again the cooling of the mucosa of the bronchial tree is a triggering factor. Bronchodilators protect against the bronchial obstruction as induced in the provocation tests.

BRONCHIAL OBSTRUCTION PROVOCATING AGENTS

In the classical sense these can be differentiated as

a. Bronchospasmogens, causing bronchial smooth muscle contraction. Examples are acetylcholine and related agents, histamine, α-adrenergic agents and SRS-A.
b. Stimulants for vagal sensory nerve endings in the bronchial mucosa, such as local cooling and possibly also certain mediators and irritants.
c. Specific antigens and possibly irritants (e.g. histamine releasing agents) acting by release of mediators from the mast cells which may act as bronchospasmogens, directly or by stimulation of the sensory vagus as well as on the bronchial mucosa as such causing local reactions like vasodilatation, edema, swelling, etc.
d. Irritants examples of which are ozon, SO_2, local pH-changes and air pollutants in general which may act like the agents mentioned under b and c.

BRONCHIAL OBSTRUCTING AND BRONCHODILATING AGENTS: LOCATION OF THEIR SITES OF ACTION

Recent physiological and pathophysiological observations enforce a change in the views on the sites of action of bronchial smooth muscle-spasmogens and -spasmolytics (Ariëns, 1980).

Blockade of the impulse-conduction in the vagal sensible and/or motor nerve fibres by means of local anesthetics, local cooling, ganglionic blockers or cutting the nerve, prevents bronchospasm such as induced by exposure to the various provocating agents. Application of agents like histamine via a catheter at only one side of the bronchial tree (one lung) brings about bronchoconstriction at both sides. Newhouse and Ruffin (1978) report that application of the provoking agent histamine at the

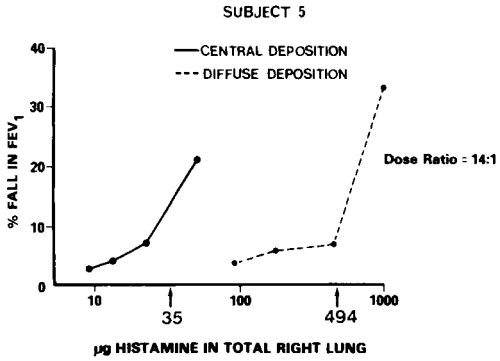


Fig. 1.

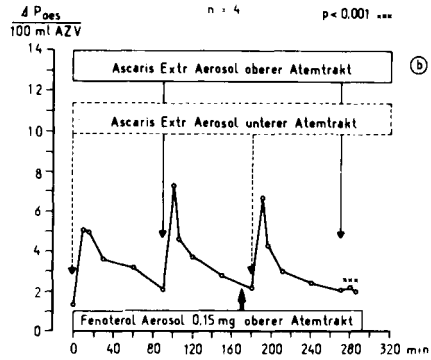


Fig. 2.

Fig. 1. Histamine aerosol dose-effect curves for central (higher airways) and diffuse (smaller airways) deposition in one subject. The numbered arrows along the abscissa represent the mean dose (5 subjects) of histamine producing a 20% reduction of FEV_1. The dose ratio for both ways of application is 14. After Newhouse and Ruffin (1978).

Fig. 2. The induction of airway obstruction (ΔP_{oes}/100 ml AZV) by Ascaris-type antigen in the sensitized dog and the effect thereon of the β-adrenergic bronchodilator fenoterol applied into the trachea (oberer Atemtrakt) and into the lower airways (unterer Atemtrakt) respectively. Note that the bronchial obstruction induced by the antigen after application into the trachea is effectively antagonized by the bronchodilator agent applied into the trachea. After Zimmermann and others (1980).

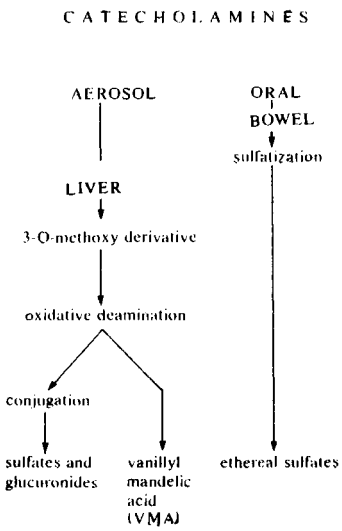


Fig. 3. Metabolic conversion of catecholamines in dependence on the route of application.

TABLE 1. Isoprenaline metabolites in urine (%)

Application	oral*	inhalation*	intrabronchial**
Metabolite			
Sulphate conjugate	93	94.9	6.3
3-OCH_3 isoprenaline	8.4	0.8	6.2
Sulphate of 3-OCH_3 isoprenaline	1.9	4.2	59.7

After Dollery, Davies and Conolly (1971)* and Blackwell and others (1974)**

end of an inhalation such that only the higher (larger, central) airways are reached appears to be 14-fold more effective in increasing the bronchial resistance than in case of inhalation with which the histamine also reaches the peripheral smaller airways (Fig. 1). In the case of exercise-induced asthma and bronchial obstruction induced by a cold aerosol of water, by the time the second bifurcation in the bronchial tree is reached, the air inhaled is saturated with water and the aerosol has reached body temperature. These observations indicate that the provoked bronchial obstruction is induced to a major extent at the level of the mucosa in the larger airways such as the trachea which implies a vagal-reflex nature. The "bronchospasmogens" including the mediators released from the mast cells apparently act as stimulants of sensory nerve endings in the mucosa thus inducing bronchial smooth muscle contraction via a reflex pathway constituted by the sensory (afferent) and motor (efferent) vagal nerve fibres.

In asthmatic patients there is not a true hyperreactivity of the bronchial smooth muscle. The sensitivity thereof for various spasmogens is hardly increased. There is, however, a strong increase in sensitivity of the mucosa of the bronchial tree for a whole scala of provoking agents. This indicates that rather a hypersensitivity on the level of the - to some extent chronical - slightly inflamed bronchial mucosa in the higher airways is involved. In this concept there is little room for bronchial smooth muscle spasmogens and spasmolytics. Also for the latter agents a site of action at the level of the bronchial mucosa in the higher airways is likely.

Recently direct evidence was reported for the mucosa of the higher airways as the site of action for "bronchospasmolytics" by Zimmermann and others (1980). Use of a bronchial catheter with at the level of the bifurcation in the trachea a balloon, allowed selective application of aerosols with provoking agents and bronchodilators into the trachea or into the lower airways. Bronchial obstruction induced in the trachea was effectively antagonized by β-adrenergic agents also applied there (Fig. 2), whereas systemic application of higher dosages of the β-adrenergic agents was not effective. Their conclusion is: "Für die Klinik ist wichtig, dass die Erfahrung am Patienten, nach der die Bronchodilatation schon im oberen Bereich des Respirationstraktus hervorragend auslösbar ist, experimentell belegt werden kann, wobei eine Wirkung lokal weitab der für den Bronchospasmus verantwortlichen Bronchialmuskulatur anzunehmen ist, die sicher auch nicht resorptiv-systemisch zustande kommt."

From studies on deposition of aerosols, after inhalation, especially in asthmatic patients (Newhouse and Ruffin, 1978) and from metabolic and pharmacokinetic studies on isoprenaline after inhalation, it may be concluded that even under optimal conditions as far as the composition of the aerosol (particle size etc.) and its application are concerned, only a minor fraction, 5% or less, of the dosis applied reaches the peripheral airways. In fact, 90% or even more of the inhaled dosis is absorbed via the intestinal tract. A high degree of sulphate conjugation indicates absorption via the intestinal mucosa; a low extent of methoxylation indicates non-intestinal absorption (Fig. 3 and Table 1).

REVISION OF THE CLASSICAL CONCEPT ON THE MECHANISM OF ACTION OF BRONCHIAL SMOOTH MUSCLE SPASMOGENS AND SPASMOLYTICS

a. In the case of asthma there is not a hyperreactivity of the bronchial smooth muscle system, but much more a hyperirritability or hypersensitivity at the level of the mucosa in the higher airways. Stimuli that normally do not or hardly induce a vagal reflex, under these circumstances may bring about enhanced vagal reflex bronchospasm. One may compare this condition of hyperirritability with the hyperalgesia known after first grade burns of the skin, where, although there is no pain, at the slightest touch, be it sensory or caloric, strong pain and muscular reflex reactions are induced.

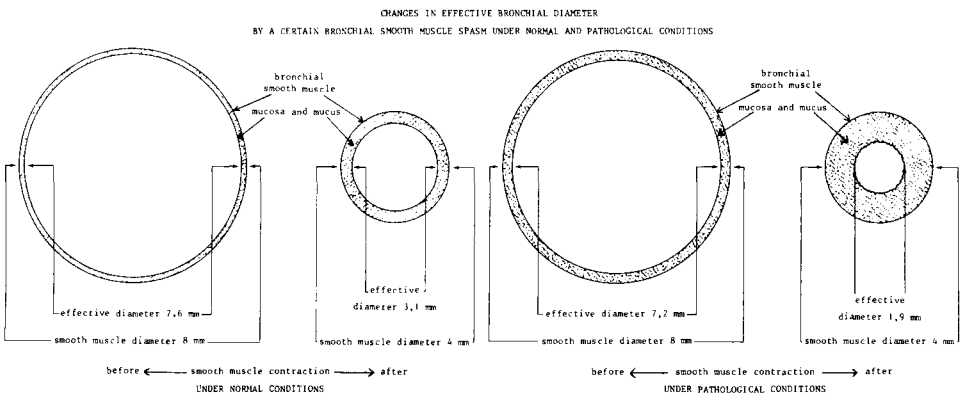


Fig. 4a-b. Schematic representation of the reduction in the effective bronchial diameter caused by a given degree of bronchial smooth muscle spasm under normal (Fig. 4a) and under pathological (Fig. 4b) conditions. Note: under normal conditions a reduction in the bronchial diameter on the smooth muscle level ("smooth muscle diameter") to 0.5 its original value results in a reduction in the "effective diameter" of 0.4 its original value. Under pathological conditions, i.e., with mucosal swelling (doubling its thickness) the same reduction in "smooth muscle diameter" results in a much stronger reduction in the "effective diameter", namely 0.26 its original value. In conclusion, the same degree of bronchial smooth muscle spasm decreases the effective diameter and therewith increases the resistance in the systems much more under pathological than under normal conditions of the mucosa. After Ariëns (1980).

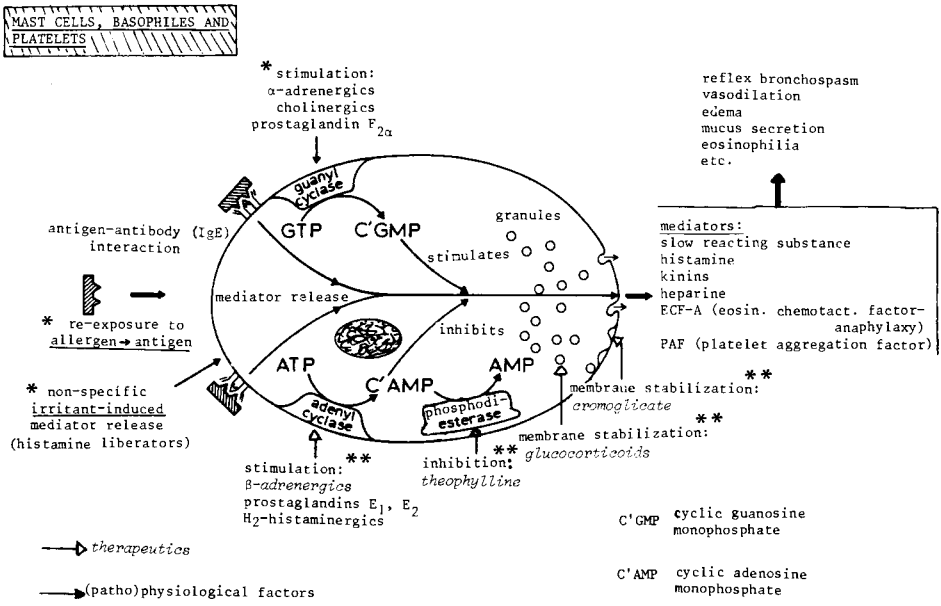


Fig. 5. Schematic representation of the sites of action on mast cells of agents enhancing* and agents inhibiting** mediator release. Modified after Webb-Johnson, Chir and Andrews (1977).

b. One has to be well aware of the fact that with respect to bronchial obstruction in asthmatics, in the increase in resistance in the airways, the condition of the bronchial mucosa can be a predominant factor, such that even a normal, non-pathological, vagal reflex bronchoconstriction can bring about severe bronchial obstruction. In the case of a modest thickening of the bronchial mucosa due to mediator and/or irritant induced vasodilatation, local edema, mucus gland hypertrophia, and mucus secretion, a certain decrease in the circumference at the level of the smooth muscle - the periphery of the bronchial wall - will, as compared to the case of a normal mucosa, bring about a greatly enhanced increase in airway resistance (Fig. 4a-b) (Ariëns, 1980). This implies that even without any excess bronchoconstriction, in the case of irritation of (slightly inflamed) bronchial mucosa, a nearly normal reflex vagal bronchial smooth muscle contraction may bring about an appreciable broncho-obstruction. The sites of action of the bronchial smooth muscle spasmogens and spasmolytics appear to be located in the mucosa of the higher airways (Scheme I). It may concern:
a. irritant receptors at the sensitive afferent vagal nerve fibres;
b. irritant receptors on the bronchial mucosa itself involved in congestion (swelling) and increased mucus production;
c. irritant receptors and antigen receptors on the mast cells involved in the release of mediators, which act then on the sites mentioned under a and b;
d. temperature sensitive sensory nerve endings detecting local cooling.

Attention will be focussed now on the β_2-adrenergic and the anticholinergic agents.

β-ADRENERGIC RECEPTORS IN BRONCHIAL ASTHMA

The mast cell is sensitive to β_2-adrenergic agents. The differentiation between β_1- and β_2-adrenergic sensitivity of organs correlates well with their sensitivity to noradrenaline and adrenaline and thus with the differentiation between the noradrenaline (neurotransmitter) receptors at the sympathetic nerve endings and the adrenaline (hormone) receptors on various tissues (Ariëns and Simonis, 1976a)(see Table 2). The mast cells have no sympathetic innervation. They are sensitive to the hormone adrenaline and thus to β_2-adrenergic agents (Heyden, van der and Zaagsma, 1980). These agents act by generating cyclic AMP that inhibits mediator release (Fig. 5) (Webb-Johnson, Chir and Andrews, 1977). They thus bring about a bronchodilatation but not by a "bronchospasmolytic" action.

An interesting aspect of the β-adrenergic receptors are the changes that take place in the receptor density on tissues, e.g. leukocytes and lymphocytes under the influence of lasting exposure to the drugs concerned. β-Adrenergic agents bring about a "down regulation" i.e. a reduction in the β-adrenergic receptor density (Fig. 6) (Galant and others, 1978). β-Adrenergic blocking agents bring about an "up regulation" i.e. an increase in the β-receptor density which in fact is a counteracting of the down regulation by the endulgous adrenergic agent adrenaline(Baxter and Funder, 1979).The down regulation may be involved in the development of resistance against β-adrenergics.The up regulation may be responsible for the withdrawal (rebound) effects as observed after stopping treatment of, for instance, angina pectoris with β-adrenergic blockers. Taken into account the hyposensitivity of the sympathetic system after β-adrenergic agents and the hypersensitivity after β-adrenergic blocking agents, one might assume that partial agonists, holding the middle between them, might not cause such changes. This then would imply that for β-adrenergic blocking therapy, agents with a certain residual intrinsic activity and that for β_2-adrenergic, e.g. bronchodilatory therapy, compounds with an intermediate (not full) intrinsic activity might be advantageous. The intrinsic activity of β-adrenergic agents is particularly dependent on the structure of the ring system in the molecule (Ariëns, 1967). Suitable manipulation, e.g. chlorine substitution therein - the prototype β-adrenergic blocker dichloro-isoproterenol (DCI) is an example - may

Biological object	Animal	Relative activity epin./norepin.
duodenum	rabbit	0.3
atrium (freq.)	guinea pig	0.7
atrium (force)	guinea pig	0.8
trachea	guinea pig	10
uterus	rat	103
lactic acidemia	rat	145

TABLE 2.

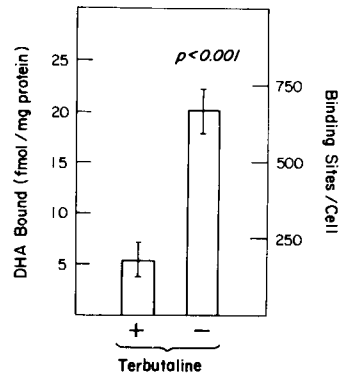
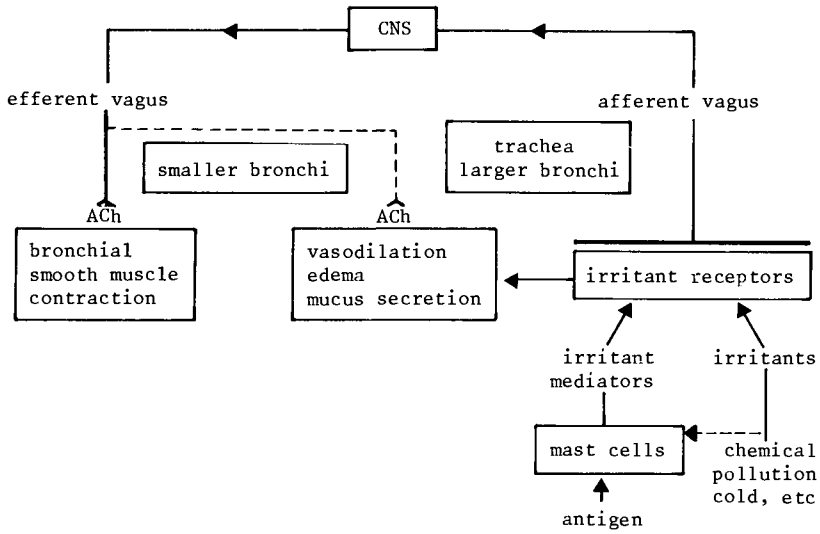


Fig. 6

TABLE 2. Relative sensitivity of various organs to epinephrine and norepinephrine. Note that the relative sensitivities correlate well with the classification of the various organs as far as their sensitivity to β_1- and β_2-adrenergic agents is concerned. After Grana, Lucchelli and Zonta (1974).

Fig. 6. Down-regulation of β-adrenergic receptors (measured by dihydroalprenolol (DHA) binding under the influence of exposure to terbutaline in patients (5) with moderate to severe asthma. 7 Days after discontinuation of the drug (-) the β-receptor density returns to normal values. Mean ± S.E.M. is given. After Galant and others (1978).



Scheme I. Schematic representation of a new concept for the mechanisms involved in the provocation of bronchial obstruction by antigens, mediators (acetylcholine, histamine and SRS-A), chemical pollutants and cooling, finding their sites of attack in the tracheal and bronchial mucosa.

result in the compounds desired. In fact, the bronchodilator clenbuterol is modified in this sense and indeed behaves as a partial agonist if tested as a functional antagonist of the spasmogen acetylcholine on isolated bronchial smooth muscle (Fig. 7a-b) (Ariëns and Simonis, 1976b; O'Donnell, 1976) and on the isolated atrium (Engelhardt, 1976). Clinically it is an effective bronchodilator (Baronti, Grieco and Vibelli, 1978; Brusasco and others, 1978). A study of the up or down regulation in the β-adrenergic receptor capacity by and of the tendency to develop resistance against the partial agonist clenbuterol as compared to full agonists - β_2-adrenergic agents in use as bronchodilators - is appealing.

β- AND α-ADRENERGIC RECEPTORS IN BRONCHIAL ASTHMA

Particularly interesting are the results of the study of β- and α-receptor density in asthmatic patients. Szentivanyi, Heim and Schultze (1979) report that in such patients the β-adrenergic receptor density - besides the down regulation reported after exposure to β-adrenergic agents - is reduced and the α-receptor density is concomitantly increased such that the balance between β- and α-adrenergic receptor density is changed in the advantage of the latter (Table 3). In the reduction in the β-adrenergic receptor density in asthmatics possibly an autoimmune component is involved (Venter, Fraser and Harrison, 1980). Experimentally, stimulation of the α-adrenergic receptors particularly during β-adrenergic blockade brings about bronchial obstruction (Snashall, Boother and Sterling, 1978) which can be antagonized by α-adrenergic blocking agents (Patel, 1979).

With regard to the disbalance between β- and α-adrenergic receptors in asthmatic patients, the question arises whether a combined β-adrenergic stimulation and α-adrenergic blockade may be favourable in anti-asthmatic therapy.For designing such a compound one can make a comparison with the compound labetalol, a β-adrenergic blocking agent with also an α-adrenergic blocking action. As is known from earlier studies on structure-action relationship in noradrenaline derivatives, substitution of aralkyl groups on the amino function results in a reduction of the α-adrenergic intrinsic activity with a maintenance of affinity and a maintenance of β-adrenergic activity and affinity (Table 4) (Ariëns, 1967), such that β-adrenergic agents with an α-adrenergic blocking action are obtained. The balance is, however, strongly in favour of the β-adrenergic action. There is no reason why not also in this case like in the case of labetalol a better balance between the two components in the action would be obtainable (Fig. 8). Such agents might be useful in the treatment of asthmatic broncho-obstruction, although as a matter of fact a combination of a β-adrenergic agent and an α-adrenergic blocking agent, undoubtedly would be feasible too.

CHOLINERGIC RECEPTORS IN BRONCHIAL ASTHMA

There is no doubt that anticholinergic agents, atropine etc., if applied systemically can block the cholinergic transmission at the vagal nerve endings and thus act as bronchospasmolytics. The quaternized anticholinergic tropinol-derivative ipratropium (Atrovent^{R}), for instance, is active in dosages of 20 μg by inhalation. Of this dosage only a minor fraction comes to absorption so that the plasma concentrations reached are much too low to cause a systemic anticholinergic action. One has to take into account that in the case of inhalation, the major part of the dose only reaches the higher airways where it is dissolved in a very thin liquid film covering the mucosa, an extremely small volume. This implies that even although very low dosages are used, still relatively high concentrations are reached on the mucosa of the higher airways. There is good evidence that mast cells are sensitive to acetylcholine (10^{-10} M) that enhances mediator release (Fig. 5) (Webb-Johnson, Chir and Andrews, 1977) an action antagonized by anticholinergics (Fantozzi and

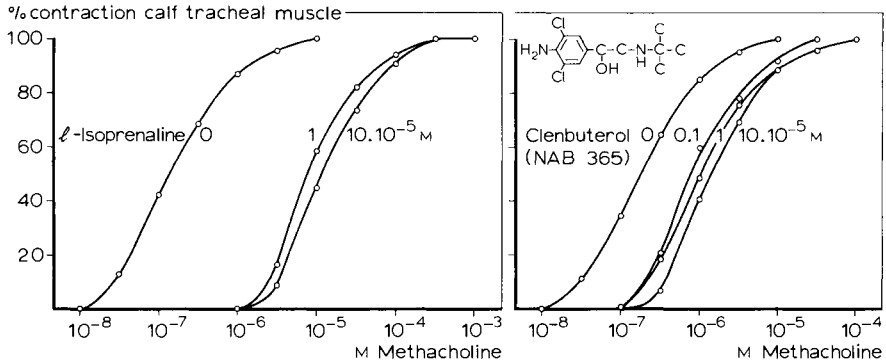


Fig. 7a-b. Isoprenaline (Fig. 7a) and NAB-365 (clenbuterol; Fig. 7b) tested as functional antagonists against methacholine on the calf tracheal muscle. Note that the maximal shift brought about by isoprenaline is much larger than that for NAB-365 which therefore behaves as a partial agonist. After Ariëns (1976b).

TABLE 3. Ratios for β-receptor/α-receptor densities in tissues of healthy individuals and asthmatic patients calculated on the basis of binding studies with H^3-dihydroalprenolol to β-receptors and with H^3-dihydroergocryptine to α-receptors.

individuals	lymphocyte membranes	lung tissue membranes
healthy	5.57	7.18
asthmatic	0.93	0.81

After Szentivanyi (1979).

HOMOLOGUES SERIES OF SYMPATHETIC DRUGS ON α- AND β-RECEPTORS (9; 11; 13a)

HO, HO, OH, H, C-C-N-R (*dl*-compounds)

R	α-receptors (ductus deferens)			β-receptors (tracheal muscle)		
	α	pD_2	pA_2	α	pD_2	pA_2
-H	1	5.4	–	1	5.8	–
-C	1	5.7	–	1	6.7	–
-C(C)C	0.4	3.4	–	1	7.5	–
-C(C)(C)C	–	–	< 2.5	1	7.6	–
-C(C)(C-ϕ)C	0	–	5.5	1	8.1	–
-C(C)C-C-ϕ	0	–	5.2	1	8.2	–

TABLE 4. Influence of aralkyl substitution in the amino function of norepinephrine (R = -H) on α-adrenergic and β-adrenergic action. Note the loss of α-adrenergic intrinsic activity with maintenance of β-adrenergic activity and increase in β-adrenergic potency as a result of the aralkyl substitution. After Ariëns and Simonis(1960).

others, 1978). The question arises why there should be cholinergic receptors on the mast cells since there is no cholinergic innervation on these cells and acetylcholine is highly vulnerable to the omnipresent choline-esterase.

An interesting aspect of the anticholinergic bronchodilator ipratropium is that in the series of alkyl substituted tropinol derivatives to which it belongs for the alkyl substitution on the amino function there are two position isomers. It turns out that the extension of the alkyl group is better tolerated in one of the two positions than in the other, probably related to the sterical conformation of and hinderance on the anionic counterpart of the onium group on the receptors (Ariëns and Simonis, 1976b). Larger substituents such as propyl and butyl are not tolerated in any of the two positions, a methyl group is well tolerated in both positions. For the isopropyl derivative, there is a ratio of about 200 for the anticholinergic action of the two position isomers (Fig. 9) (Wick, 1972). Ipratropium, the more potent of the two, also appears to be the more potent bronchodilator (a ratio of about 70) as measured in inhalation tests in dogs (Engelhardt, 1980). Experiments as presented in Fig. 2 but then with ipratropium may produce more definite evidence for a bronchodilatory action induced therewith on the level of the mucosa of the higher airways. Then the question must be answered whether cholinergic receptors on the mast cells really are the sites of action for the bronchodilatory anticholinergics.

With the emphasis on the reflex nature of bronchospasms and thus the role of sensory vagal nerve endings, the question arises whether compounds with a local anesthetic action might be of use as bronchodilators.

LOCAL ANESTHETICS AND BRONCHODILATION

Undoubtedly, local anesthetics applied on the bronchial mucosa block bronchial obstruction induced by irritation or stimulation of the mucosa (Sampson, 1977). A problem is that most local anesthetics have some irritant action before the anesthetic action becomes manifest. This may be due to the fact that these compounds, tertiary amines, readily penetrate into the various tissues and cells. Local anesthetic action as such, however, requires an action of the agents (amines) in the protonated form. At the pH in the tissues they must be present partially in a non-ionized form in order to penetrate the myeline sheath surrounding the nerve fibres and partially in the protonated form, in order to perform the local anesthetic action. In the sensory nerve endings, however, the nerve fibres (axons) are not covered by a myeline sheath, such that here quaternary onium compounds with local anesthetic potency will be active anyway. One could consider quaternized derivatives of the known local anesthetics as potential bronchodilator agents to be used by inhalation. The anesthetic action in the buccopharyngeal area as well as the possible bitter taste of such quaternary derivatives may be prohibitive for their use as inhalant.

The aim of this presentation was to take, on the basis of recent experimental evidence, a critical stand with regard to certain well established views in the field of anti-asthmatic therapy. Some classical concepts had to give way to this evidence and thus were replaced by new concepts that may open perspectives for new lines of approach in the development of anti-asthmatic agents.

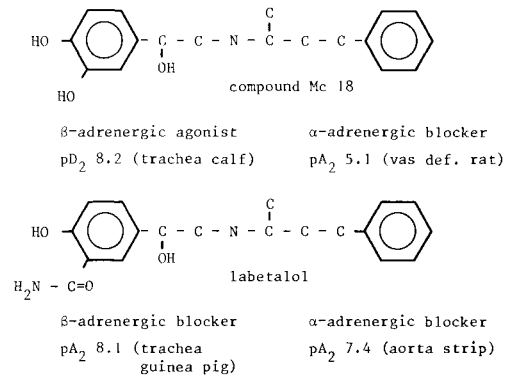


Fig. 8.

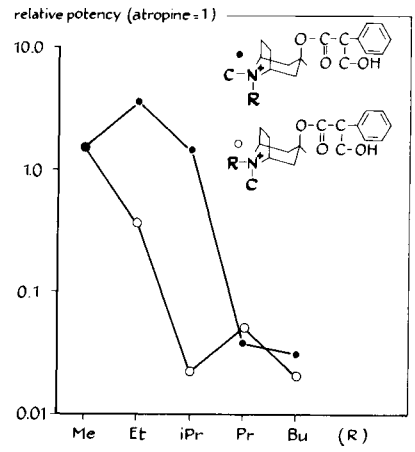


Fig. 9.

Fig. 8. Structure-action relationship for the β-adrenergic agonist - α-adrenergic blocker Mc 18 and the β-adrenergic blocker - α-adrenergic blocker labetalol. Introduction of the aralkyl substituent brings about the elimination of the α-adrenergic intrinsic activity (see Table 4); modulation of the cathecholamine moiety results in a reduction (see Fig. 7b) or elimination of the β-adrenergic intrinsic activity.

Fig. 9. Relative potency in two series (geometric isomers) of N-alkylsubstituted atropine derivatives tested as antagonists of acetylcholine on the guinea pig ileum. Note that the tolerance for alkyl substitution is larger in one (•) of the series. For the isopropyl (iPr) derivative the ratio for the anticholinergic activities is about 200, the more active isomer being ipratropium. After Wick (1972).

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Agents Acting on the Immunological Component in Respiratory Diseases

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ABSTRACT

Disodium cromoglycate (DSCG) was introduced in 1969 for the prophylaxis of bronchial asthma and a variety of allergic conditions. Since then, intense efforts have been made to develop other compounds which inhibit the release of histamine and SRS-A from sensitized mast cells but which are orally effective. Recent progress will be reviewed and chemical structure-biological activity relationships discussed.

The synthesis of a series of (adamantylcarboxamido)-phenylalkanoic acids is described. The antiallergic effect of 2-(1-adamantylcarboxamido)phenylacetic acid (GPA 2476) is measured in guinea pigs and ascaris sensitized dogs. The clinical findings with GPA 2476 will be presented.

KEYWORDS

Disodium cromoglycate, asthma, allergy, histamine, SRS-A, adamantylcarboxamidophenylacetic acid, GPA 2476.

INTRODUCTION

"If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts, he shall end with certainties." (Francis Bacon – 1605.)

Seldom has the pharmaceutical industry mounted such a sustained effort in pursuit of a therapeutic goal with as little commercial success as the area of antiallergic drugs. The presentation will be divided into (i) the historical development of antiallergic agents which have as their chief mechanism of action the inhibition of mediator release. and (ii) the synthesis and development of a series of adamantylcarboxamidophenylacetic acids as potential antiallergic drugs.

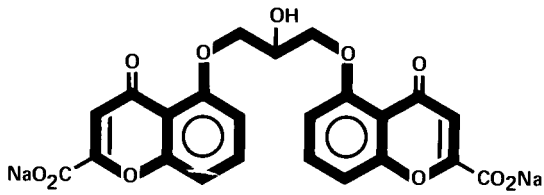
HISTORICAL DEVELOPMENT OF INHIBITORS OF MEDIATOR RELEASE

The stimulus which resulted in considerable efforts in many pharmaceutical companies was the introduction of disodium cromoglycate (Fig. 1) (DSCG) by Fisons in 1969 for the prophylactic treatment of bronchial asthma.

DSCG itself represented the culmination of considerable efforts by Fisons starting with the observation that khellin, a naturally occuring chromone and a specific muscle relaxant, had been used also for the relief of bronchial asthma but suffered from the side effects of nausea/vomiting. The progression of events that led to the development of DSCG has been reviewed by Cox (1970). So, for the purposes of historical

perspective, the launching of DSCG will be the starting point for the presentation.

Figure 1



(I)
disodium cromoglycate (DSCG)
FPL 670 (Fisons)
(PCA 0.8 i.v., inactive p.o.)

DSCG was certainly a 'breakthrough' drug in the prophylaxis of immediate anaphylactic reactions. Bronchial asthma can be considered as a local manifestation in the lung of an immediate type anaphylactic reaction and an appropriate animal model is the Passive Cutaneous Anaphylaxis (PCA) in rats, which is widely used throughout the pharmaceutical industry as a primary screen in this area. DSCG is accepted to have an important place in the prophylactic amelioration of allergic bronchial asthma and had no bronchodilator, antiinflammatory or antihistaminic activity but had, as its principal action the inhibition of the release of spasmogens (histamine, SRS-A) from mast cells. This unique property offered a novel approach to the management of respiratory diseases in which an allergic mechanism may be contributing to the condition and includes allergic bronchitis and bronchial asthma. In addition to the fact that it was the first prophylactic treatment of asthma, the chief advantages of DSCG therapy were: (i) reduction in symptoms of asthma (sneezing and coughing), (ii) a reduced need for oral bronchodilators, aerosolized adrenergics and corticosteroids, (iii) a greater exercise tolerance and (iv) the compound was remarkably non-toxic and free of side effects.

Thus, DSCG had considerable interest and activity. To counterbalance the favorable factors, there are also some very specific problems or disadvantages with DSCG: (i) the relatively modest number of patients to whom the drug is of clinical benefit (30-50%) – particularly in the United States, (ii) the mode of administration. Since DSCG is inactive when administered orally, it is given by insufflation using a Spinhaler.® A recent development is the introduction of "pelletized" Intal® which consists of pure DSCG pellets, which, when inhaled, break down into the particle sizes required in the lung, thus abolishing the previous requirement for lactose and providing an improved delivery form. Therefore, with the stage set by DSCG the goal for the pharmaceutical industry was clear: the development of a compound with a biological profile like DSCG but one which was orally effective. From this simple goal has come several years hard work by many groups throughout the world, and as yet, no other anti-allergic drug which inhibits mediator release as its chief mechanism of action has been marketed, although hopefully that day will soon arrive. I will attempt to present an illustration of the thinking of medicinal chemists as they approach the goal mentioned above.

The majority of compounds that exhibit inhibition of mediator release from mast cells have a common structural element which is depicted in Fig. 2.

Figure 2

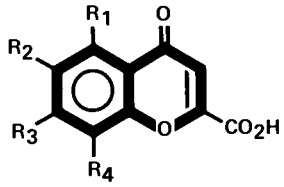
ARYL – X – Y – Z

1. Aryl or heteroaryl
2. X = heteroatom (O,N,S)
3. Y = double bond, $\underset{\underset{O}{\|}}{C}$, or aryl
4. Z = CO_2H or equivalent.

Common structural element in inhibitors of mediator release.

CHROMONES

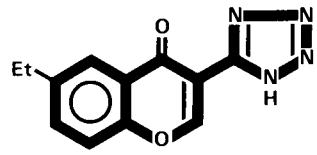
The most closely related compounds to DSCG are obviously those possessing a chromone nucleus. Representative examples are shown in Fig. 3 indicating their activity in the PCA screen (mg/kg) together with their name/code number, where available, and also the company from which they emanate.



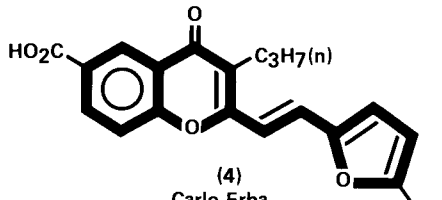
(2)

a) R_1R_3=H, R_2,R_4=C(Me)$_3$
Terbucromil (PCA 0.1 i.v., 4.2 p.o.)

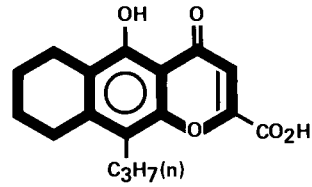
b) R_1=OH, R_2,R_4=Et, R_3=H
FPL 52757 (PCA 5.0 i.v., 5.0 p.o.)
(Fisons)



(3)
AA–344
(PCA 0.29 i.v., 6.9 p.o.)
(Takeda)



(4)
Carlo Erba
(PCA 10 p.o.)



(5)
Proxicromil (FPL 57787)
(PCA 6.4 i.v., 15.5 p.o.)
(Fisons)

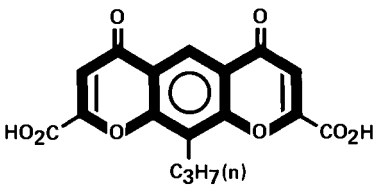
Figure 3 – Chromones

In addition to its mediator release properties, terbucromil, 2a has been found to possess uricosuric effects in man (Augstein, 1977). AA. 344, 3 inhibited histamine release from isolated rat peritoneal mast cells mediated by immunoglobulin E or immunoglobulin Ga-antibodies, or histamine induced by chemical agents such as concanavalin A, dextran and compound 48/80 (Kuriki, 1977). 3 is metabolized to 3 major metabolites, the 2-N glucuronoside, the 6 CH_3CHOH and 6 CH_3CO (for ethyl). These latter compounds were 0.5 x and comparably active orally in PCA with AA-344 itself (Nohara, 1979; Kito, 1978). 4 shows a potency comparable with DSCG and exhibits self-tachyphylaxis in vitro and in vivo as does DSCG (Doria, 1979).

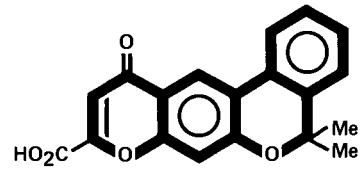
The most promising of this series of chromone derivatives is proxicromil 5 which was also effective in inhibiting the antihuman, IgE-induced release of histamine and degranulation of human basophils, whereas DSCG is inactive in this test. In experimental bronchial provocation tests in man, 5 is effective either by inhalation or orally at doses of 4-10 mg. The earlier chromone derivatives terbucromil 2a and FPL 52757 2b are also active in these tests. 5 is presently in clinical trial and has shown efficacy in allergic skin conditions, and in a double blind study in 10 patients prevented exercise-induced asthma at a dose of 12 mg orally (Thomson, 1980).

Metabolism of 5 in rat, dog, hamster, rabbit, monkey and man is primarily by hydroxylation of the alicyclic ring. In dogs, the rate of hydroxylation is much slower than in other species resulting in saturation of biliary excretion of 5 and severe hepatotoxicity at doses of 60 mg/kg/day (Smith, 1979). This hepatotoxicity was noted only in the dog, and hopefully proxicromil will be introduced in the U.K. by the end of 1981.

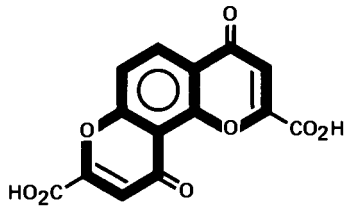
As a first major modification the chromone skeleton was retained intact but incorporated into polycyclic ring systems as shown in Fig. 4.



(6)
(PCA 0.18 i.v.)
(Fisons)



(8)
PRD–92–EA
(PCA 0.5 i.v., 21.8 p.o.)
(Pharma Res.)



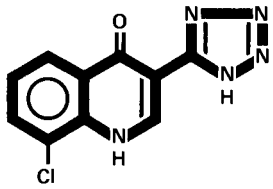
(7)
(PCA 7.6 i.v.)
(Fisons)

Figure 4 – Tricyclic Chromone Analogs

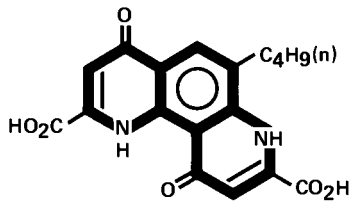
6 when given as an aerosol of its calcium salt to man was effective against asthma, hay fever and uticaria with a longer duration of action than DSCG. SAR studies have indicated that the linear analogs are more active than the corresponding angular analogs 7 (Bantick, 1976). PRD-92-EA 8 represents the best of a series of tetracyclic chromone derivatives with good activity in PCA in animals i.v. In addition, 8 also prevented antigen-induced bronchoconstriction in Rhesus monkeys (Devlin, 1977).

QUINOLONES

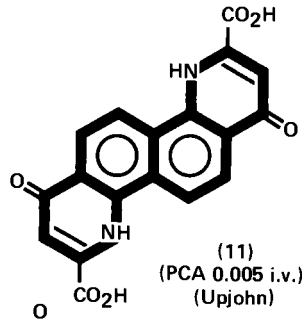
To the medicinal chemist the next logical step is the replacement of one heteroatom (O), present in the chromones, by another (N) to yield quinolones as shown in Fig. 5.



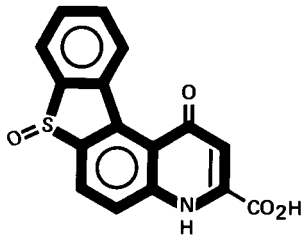
(9)
(PCA 0.078 i.p.)
(Riker)



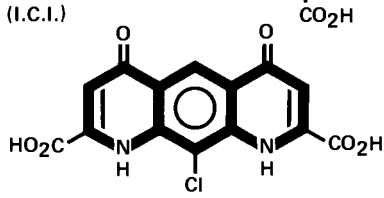
(10)
ICI–74917 – bufrolin
(PCA 0.005 i.v.)
(I.C.I.)



(11)
(PCA 0.005 i.v.)
(Upjohn)



(12)
(PCA 0.1 i.v.)
(Riker)



(13)
U–38650
(PCA 0.05 i.v., 25 p.o.)
(Upjohn)

Figure 5 – Quinolones

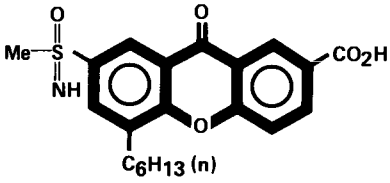
The tetrazolyl quinoline 9, after appropriate reduction of the particle size was effective at 0.156 mg/kg p.o. (Erickson, 1979). The condensed quinoline derivatives illustrated in Fig. 5 are some of the most potent inhibitors in the PCA screen. Perhaps the best known is 10 bufrolin (ICI 74917). 10 is 5 x DSCG as a phosphodiesterase inhibitor and approximately 100 x DSCG in the PCA model (Barrett-Bee, 1976). In normal guinea pigs and in an isolated lung preparation 10 provoked a mild bronchospasm, but this does not occur in any of 7 other species, including man (Sheefer, 1975). However, although 10 was shown to be effective in allergen induced bronchoconstriction in asthmatic patients (Church, 1978) and afforded almost complete protection against nasal stenosis induced by grass pollen extracts (Vilsvik, 1976) it failed to improve the symptoms of asthma over a 4-week period (Ellis, 1978) and has been abandoned clinically. The fused bisquinoline carboxylic acid 11 showed activity in PCA comparable to bufrolin (Hall, 1977) but was not pursued by Upjohn due to the development of the oxamic acids which will be discussed later.

The tetracyclic quinolone-2-carboxylic acid 12 of Riker is 8 x DSCG in potency by i.v. administration but is inactive orally (Wade, 1978). U-38650 13 indicates a qualitative and quantitative advantage over DSCG in that orally it inhibits mediator release and has bronchodilator activity (Hall, 1977). 13 inhibited immediate type hypersensitivity to Ascaris antigen in Rhesus monkeys by intrabronchial administration and was 1000 x DSCG by this route.

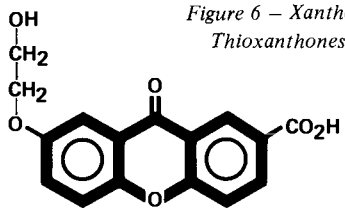
XANTHONES, THIOXANTHONES AND ANALOGS

Concurrently with the development of some of the more advanced chromone and quinolone derivatives mentioned earlier, the fertile mind of the medicinal chemist began to think in yet another well-worn path. What would happen if the chromone is expanded into its benzo analog-xanthone? In Fig. 6 a number of these are illustrated.

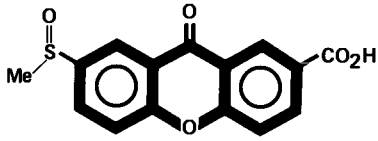
Figure 6 – Xanthones, Thioxanthones, Etc.



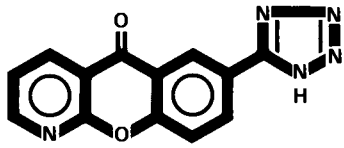
(14)
RU–31156
(PCA 0.05 i.v., 0.19 p.o.)
(Roussel)



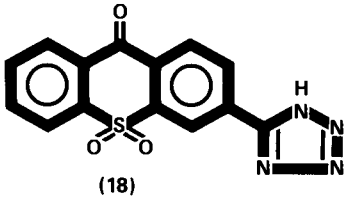
(15)
AH–7725
(PCA 0.05 i.v., 100 p.o.)
(Allen & Hanburys)



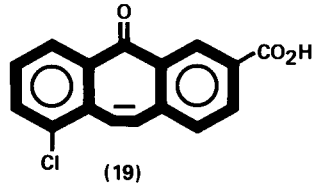
(16)
R–57337 – Tixanox
(PCA 0.05 i.v.)
(Syntex)



(17)
Y–12141
(PCA 0-1 i.v., 2.5 p.o.)
(Yoshitomi)



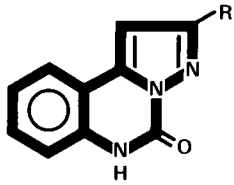
(18)
Doxantrazole
(PCA 1.0 i.v., 10 p.o.)
(Burroughs Wellcome)



(19)
(Syntex)

The earliest members of this type were 15 AH 7725 and 16 tixanox (R 57337). Both have undergone clinical trials and have been effective in exercise-induced asthma/bronchospasm when given as an aerosol (Church, 1978; Rosenthal, 1977). RU-31156 14, the most recent addition of the xanthone type, is claimed to have a potency 250 x DSCG by i.v. administration and is also effective at 0.19 mg/kg/p.o. administered as its tris-(hydroxymethyl)amino methane salt (Barnes, 1979). 14 is also 100 x DSCG against anaphylactic bronchoconstriction in rats. Like DSCG, tachyphylaxis was noted with 14 both i.v. and orally, and cross tachyphylaxis between the two indicates a similar mechanism of action (Hiller, 1978). Of the remaining related compounds, 19 is less active than DSCG (Dunn, 1979) but Y-12141 17 and the thioxanthone 18 doxantrazole show good efficacy (Goto, 1979; Wuethrich, 1976). 18 also has a potency 10 x theophylline as a phosphodiesterase inhibitor and its antiallergic activity may be due in part to this property. Clinically, at a dose of 200 mg orally 18 inhibited the immediate type asthmatic response in 8 asthmatic patients challenged with a specific antigen, e.g., house dust-mite (Hadyn, 1975; Batchelor, 1975). However, later studies of 18 at doses up to 400 mg orally showed it to be ineffective in exercise-induced asthma and Wellcome has discontinued work on this drug. (Poppius, 1977). Replacement of the tetrazole moiety of 18 by a carboxyl function results in a significant loss of activity in the PCA (Johnson, 1976).

Two additional tricyclic series are illustrated in Fig. 7.

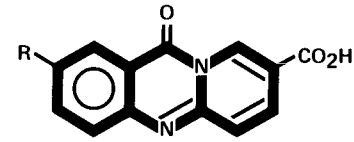


(20)

a) R=CH_2OH Pirquinazol (SQ–13847) (PCA 2–4 p.o.)

b) R = tetrazole

c) R = CO_2H (SQ–12903) (Squibb)



(21)

a) R=H (PCA 0.5 i.v., 2.5 p.o.)

b) R=Me (PCA 0.05 i.v., 0.75 p.o.) (Parke Davis)

c) R = MeO (PCA 0.2 i.v., 1.1 p.o.) (Roche)

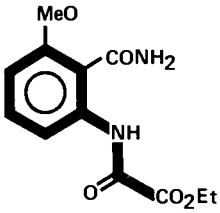
Figure 7

Pirquinazole 20a is rapidly metabolized to the corresponding carboxylic acid (SQ-12903) which is weakly active p.o. but 100 fold more potent than 20a i.v. in the PCA assay (Free, 1979; Lan, 1979). The corresponding tetrazole analogs 20b are claimed to show efficacy in PCA and also to possess antiinflammatory activity (Squibb, 1978). The series of pyrido (2,1-b) quinazoline carboxylic acids 21 show good activity both by i.v. and oral administration. In the rat allergic bronchospasm model 21b was 10 x more potent orally than doxantrazole 18 (Schwender, 1979). 21c also inhibited IgE-induced bronchospasm in the passively sensitized rat at 0.2 mg/kg/p.o. Like DSCG, 21c inhibited antigen-induced histamine release from rat peritoneal cells in vivo and failed to antagonize bronchospasm when ingested prior to i.v. histamine, serotonin, or methacholine (Tilley, 1980; Hope, 1980; Czyzewski, 1980).

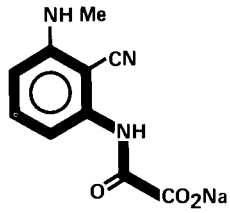
OXAMATES

A somewhat different class of compound at least at first sight is illustrated in Fig. 8, but here the common structural element may still be discerned. This class – the oxamates, has been studied extensively by Wyeth and Upjohn.

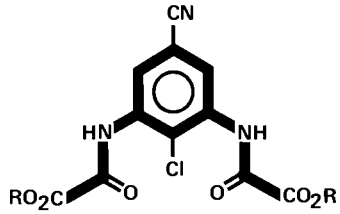
WY-16922, 22 was initially discovered as a highly active impurity in a cyclic substance (a quinazoline carboxylic acid ester) of weak activity, of which mention will be made later, and was historically the first of this series of oxamates. A significantly more potent compound, 23 has recently been described (Lewis, 1980). Of the oxamates, the most widely studied is lodoxamide 24, either as the ethyl ester or as the tromethamine salt. 24 demonstrated good oral antiallergic activity in animals and clinically given as an aerosol (0.1 or 1 mg or p.o. 1 and 2 mg); it prevented or diminished allergen-induced bronchoconstriction in 10 allergic asthmatic patients. In another trial, lodoxamide tromethamine aerosol protected 11 patients from bronchospasm induced by treadmill running (Ford, 1979; Brooks, 1979). In monkeys, 24a inhibited



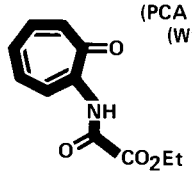
(22)
WY–16922
(PCA 5 i.v., 25 p.o.)
(Wyeth)



(23)
WY–41195
(PCA 0.3 p.o.)
(Wyeth)



(24)
a) R=Et – Lodoxamide ethyl (U–42718)
b) R=H – Lodoxamide (U–42585E)
(PCA 0.001 i.v., 5 p.o.)
(Upjohn)

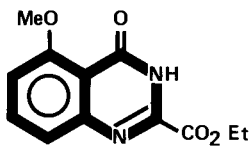


(25)
AY–25674
(PCA 5 i.v., 50 p.o.)
(Ayerst)

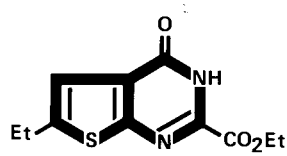
Figure 8 – Oxamic Acids

ascaris antigen-induced changes in respiration rate and tidal volume with an ID_{50}~ 1 mg/kg with a duration of ~3 hours. 24a was also effective in preventing lung function changes in guinea pigs sensitized to egg albumin (Johnson, 1979). 24a, which appeared to have fewer cardiac and gastrointestinal side effects in animals than 24b, given p.o. 1 or 3 mg prevented or diminished allergen induced bronchospasm in 2 x 12 allergic asthmatic patients. One of the side effects noted was a mild sensation of heat, a symptom also observed with some chromones (Case, 1979; Katcher, 1979). The tropone oxamate derivative AY 25674, 25 has also recently been described and was also effective in a model of passive lung anaphylaxis (antigen-induced bronchoconstriction) in the rat (Bagli, 1979; Martel, 1978).

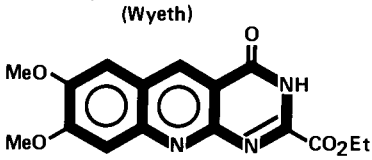
Logically, chemists presented with a new structure type and considering using it as a lead compound pursue one of two courses: (i) If the lead compound is bicyclic, open it to an appropriately substituted monocyclic system, or (ii) If the lead compound is an appropriately substituted monocyclic system, cyclize it. In Fig. 9 are illustrated various cyclic analogs of the oxamates referred to previously.



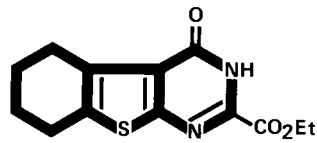
(26)
(PCA i.v. <100)
(Wyeth)



(27)
(PCA 2.4 p.o.)
(Mead Johnson)



(28)
Pirolate
(PCA 0.002 i.v., 0.3 p.o.)
(Pfizer)



(29)
(PCA >50 p.o.)
(Mead Johnson)

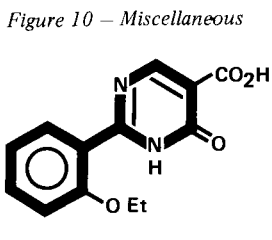
Figure 9 – Cyclic Oxamates

26 was the initial compound which led to the development of the oxamic acid derivatives described in Fig. 8. As is general with oxamate analogs, the esters are normally active p.o. and have a longer duration of action than the corresponding carboxylic acids to which the esters are rapidly metabolized. Compounds 27, 29 show some activity in PCA but are generally rather low in potency (Sellstedt, 1975; Temple, 1979). Pirolate 28 however, does show high potency in PCA, both by i.v. and oral routes (Althuis, 1980). Structure activity relationships for this series indicate that the best oral activity is found in ethyl esters with methoxy/or ethoxy groups at the 7 and 8 positions of the pyrimido [4,5-b] quinoline nucleus (Althuis, 1979).

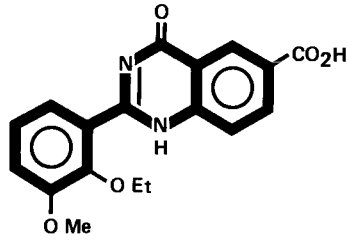
MISCELLANEOUS

A group of compounds of miscellaneous structures in which the common structural element identified in the previous series is either absent or substantially modified is illustrated in Fig. 10.

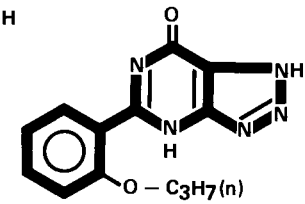
Figure 10 – Miscellaneous



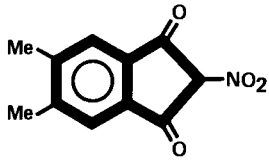
(30)
(PCA 0.07 i.v., 0.5 p.o.)
(Bristol Myers)



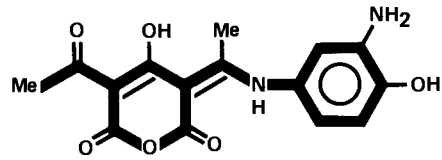
(31)
(PCA 0.15 i.v., 1 p.o.)
(Carlo Erba)



(32)
M&B–22948
(May and Baker)



(33)
BRL–10833 – Nivimedone
(PCA 0.08 i.v., 1.1 p.o.)
(Beecham)



(34)
SKF–78729–A
(PCA 0.7 i.v., 0.7 p.o.)
(Smith Kline & French)

Compounds (30-32) appear to have similar structure activity relationships since in each case the presence of an alkoxy moiety in the ortho position of the pendant phenyl ring is essential for good activity. In some respect 31 is like a 'seco' analog of the Roche and Parke Davis compounds 21 discussed previously. Another requirement includes a free NH group on the pyrimidone moiety. It has been suggested that in the case of the highly active compounds, hydrogen bonding occurs between a nitrogen atom of the pyrimidine nucleus and the ethereal oxygen (Juby, 1979; Broughton, 1975). The optimization of the azapurinones 32 was achieved by use of QSAR (Wooldridge, 1976). An additional requirement noted in 31 and in 21 is that the carboxy substituent must be in a 1-3 relationship with the carbonyl function of the condensed pyrimidinyl nucleus (Doria, 1979). M & B 22948, 32 has been claimed to inhibit both histamine and SRS-A release from human lung tissue in vitro (20 x DSCG) in addition to inhibition of histamine release from rat mast cells. 32 has some similarities to theophylline and may act through phosphodiesterase inhibition.

Nivimedone, 33 demonstrates good activity in the PCA and has been claimed to have modest SRS-A antagonism. This series has been actively pursued for a number of years and several variants of the nitro-1,3-diones have been published. The dialkyl group confers greatest activity with a minimal amount of toxicity. In asthmatic patients 33 both by aerosol (40 mg) and by the oral route (2-10 mg/kg) has shown no significant effect (Zwi, 1977; Rosenthal, 1977).

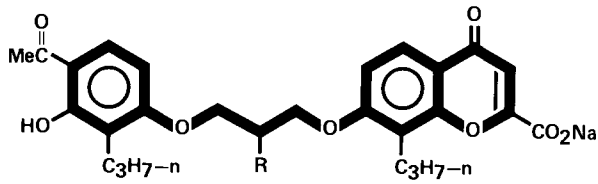
The pyranenamines, as illustrated by SKF 78729-A, 34 is a series of 3,5-diacetyltriones with activity qualitatively similar to DSCG except that it has oral activity. In the Rhesus monkey 34 at 5 mg/kg/i.v. or

by aerosol inhibited ascaris antigen provoked allergic asthma and was also active in a similar canine model (Chakrin, 1978). QSAR techniques played a major role in the development of 34 following an analysis of data which suggested that increased substituent hydrophilicity might enhance potency (Cramer, 1979).

SRS-A ANTAGONISTS

DSCG has been shown to inhibit the antigen-induced release of the mediators of allergic reaction, but it is not equally effective at inhibiting the release of all mediators. In particular, it is poor at inhibiting the release of SRS-A (slow reacting substance of anaphylaxis) when antibodies other than IgE are involved (Orange, 1971). It has been suggested that the failure of DSCG to benefit some patients with bronchial asthma might be due to the involvement of IgG antibodies, which may release SRS-A from sources other than the mast cell by a mechanism resistant to treatment with DSCG (Bryant, 1975). A substance which has the ability to antagonize SRS-A plus stabilization of mast cells may be of greater therapeutic value than DSCG in the treatment of bronchial asthma. SRS-A and histamine are important prime mediators in allergic asthmatic reaction and it was thought that metabolites of arachidonic acid were secondary mediators. However, the structure of an SRS from mouse leucocyte mastoma has been reported as a product of the lipoxygenase pathway of arachidonic acid (Murphy, 1979; Hammarström, 1980). This substance is now called leukotriene C, and has been synthesized (Corey, 1980), but, it is not yet known whether this is identical to the SRS-A released from the lung (Nijkamp, 1980).

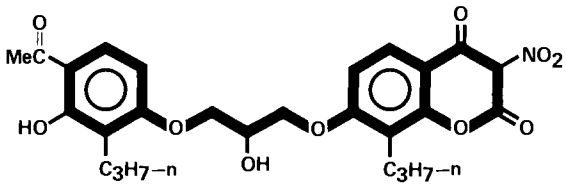
The compounds illustrated in Fig. 11 have such antagonism to SRS-A. They have much in common with each other together with their relationships to the chromones for 37 and the nitro 1,3-diones for 38.



(37)

a) R=OH FPL 55712
(SRS–A IC50 80 ng/ml)

b) R=H
(SRS–A IC50 10 ng/ml)
(Fisons)



(38)
(SRS–A ED50 0.2 μM)
(Beecham)

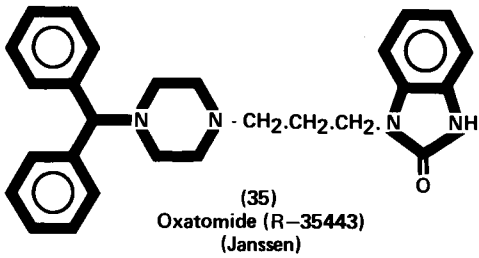
Figure 11 – SRS-A Antagonists

FPL 55712, 37 is a potent and highly specific competitive antagonist of SRS-A and has been used as a pharmacological tool for the identification of SRS-A and to help define its role in immediate hypersensitivity. Unfortunately, the compound showed a very short half-life (0.6 min. i.v. in guinea pigs) posing considerable difficulties in its in vivo use (Chand, 1979). The structural requirements for an in vitro SRS-A antagonist in compounds containing the 4-hydroxy-3-nitrocoumarin nucleus, e.g. 38 (Buckle, 1979) were similar to those previously reported for the 2-carboxy chromones (Appleton, 1977). 38 also inhibits antigen-induced histamine release in a rat passive peritoneal anaphylaxis assay when given i.p. (200 x FPL 55712 and 12 x DSCG).

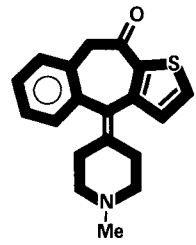
ANTIHISTAMINES

Normally, in the treatment of bronchial asthma, frank antihistamines are seldom of value, but the antihistaminic compounds which are illustrated in Fig. 12 have been claimed to be useful in the treatment of asthma.

Figure 12 – Antihistamines



(35)
Oxatomide (R–35443)
(Janssen)



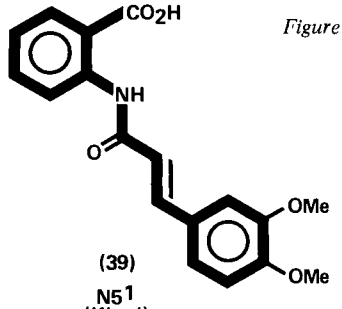
(36)
HC–20511 – Ketotifen
(Sandoz–Wander)

Oxatomide 35, in addition to being an antihistamine and antiserotonergic, also inhibits mediator release from mast cells (Borgers, 1978; Awouters, 1977). Clinically, 35 (at doses of 30-360 mg/day p.o.) was particularly effective in the treatment of hay fever, perennial rhinitis, asthma (in young patients), allergic dermatitis and was well tolerated with a low incidence of side effects (DeCree, 1980). However, 35 was found to be ineffective in the treatment of allergen-induced or exercise-induced asthma in adults (Ahonen, 1979). Ketotifen 36 has been shown in several clinical trials to be an effective antiasthmatic compound. In addition to having antianaphylactic properties, 36 is a potent H^1 histamine antagonist and a phosphodiesterase inhibitor (Wuethrich, 1978; Martin, 1978; Craps, 1980). However, 36 often causes severe drowsiness necessitating withdrawal from treatment in adult asthmatics, and the high incidence of side effects appears a high price for the marginal benefit provided with this drug (Prowse, 1980).

ANTHRANILIC ACIDS

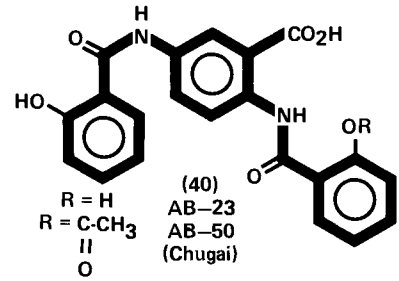
Some compounds related to anthranilic acid which have been claimed to have utility as antiallergic drugs are illustrated in Fig. 13.

Figure 13 – Anthranilic Acids



(39)
N5[1]
(Kissei)

a)
b)



(40)
AB–23
AB–50
(Chugai)

Compound 40a (AB-23) has been reported to be more active than DSCG in the rat PCA and in vitro histamine release assays (Oshugi, 1977). AB50, 40b, a prodrug for 40a is presently in clinical trial. N5', 39 is a compound of considerable interest and has been reported comparable to DSCG in the PCA in rats and rabbits. Using the PCA screen at CIBA–GEIGY 39 had minimal activity even when administered 30 min. prior to the antigen (Conroy, 1978). Nevertheless, a large number of tests have been used to examine the activity of N5' including the inhibition of degranulation of mast cells. The results concluded that N5' inhibits Type I homocytotropic antibody-mediated reactions (Koda, 1976). Unlike DSCG, 39 inhibits histamine release from human leukocytes in vivo. Clinically, it has been demonstrated that 6 weeks therapy with daily doses of 5 mg/kg/p.o. or 10 mg/kg/p.o. 39 was significantly effective in 277 children with asthma. Children with moderate asthma responded to the greatest extent (Shioda, 1979). 39 inhibited inhalation provocation and allergic skin reaction and also histamine release in 2/4 patients with mite allergy (Yui, 1979).

SYNTHESIS AND DEVELOPMENT OF ADAMANTYLCARBOXAMIDOPHENYLACETIC ACIDS AS POTENTIAL ANTIALLERGY DRUGS

An alternate title might be the story of how CIBA–GEIGY became interested in antiallergic drugs and what happened. I would like to emphasize that the work is quite old – although not previously published – and some of the biological test systems are less favored today. An up-to-date comprehensive review of biological test sysems for antiallergic drugs has been published (Herzig, 1978; Krell, 1980).

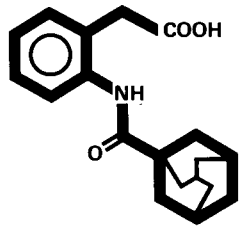
HISTORICAL

In 1967 Geigy had a non-steroidal anti-inflammatory drug in clinical trial which was unique at the time in that unlike many other compounds in this area which are strongly acidic, e.g., phenylacetic acids or enolic anilides, this compound was essentially neutral. In early clinical trials the compound demonstrated efficacy in inflammatory conditions, but an unacceptably high number of rashes occurred after about the 9th day of treatment. It was speculated that these rashes were a manifestation of a 'delayed hypersensitivity' reaction and conceivably were caused by a metabolite, a hydantoin, since these are known to cause rashes. A challenge was made to the pharmacologists to ascertain whether or not: (i) this really was a delayed hypersensitivity Rxn, and (ii) to devise a test system that would be capable of detecting at an early stage compounds which would have this potential. The test system chosen was the Jerne hemolytic plaque technique. This assay detected the potential of a compound to cause rashes, but it was also used later to identify compounds which would be selective immunosuppressants for humoral antibodies. This ability, it was argued, could have potential utility in the treatment of allergic conditions since certain humoral antibodies (IgE) are those involved in allergy. Many compounds were assayed, and the first compound to show activity was 2-(4-chlorobenzamido)phenylacetic acid. This compound showed non-steroidal antiinflammatory activity in a variety of screens (UV erythema, adjuvant arthritis, anti-carrageenin) plus moderate activity in the Jerne screen. Subsequently it was found that GPA 2476 41 2-(1-adamantylcarboxamido)phenylacetic acid was devoid of antiinflammatory activity in the usual assays, but demonstrated good activity in the Jerne screen.

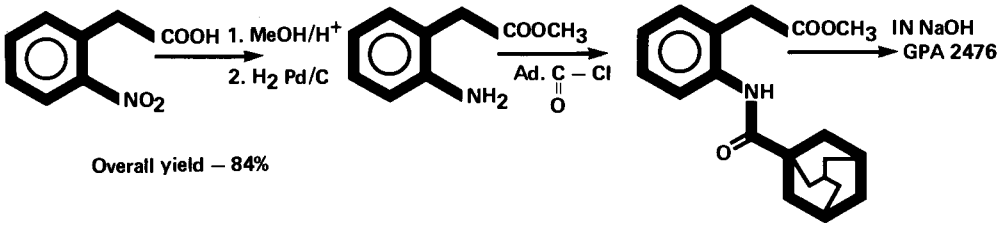
CHEMISTRY

GPA 2476, 41 was readily available by a 4-step sequence from 2-nitrophenylacetic acid in an overall yield of 84% as indicated in Fig. 14.

Figure 14



(41)
GPA 2476



Overall yield – 84%

A number of variants were prepared by standard methods and included: (i) isomers involving attachment of 1-admantylcarboxamide group at the 3 or 4 position relative to the acetic acid moiety, (ii) substitution in the phenyl ring (Me, MeO, Cl, CF_3, isopropyl, t-butyl), (iii) variation of side chain to the corresponding cinnamic and propionic acids, (iv) variation of carboxylic acid (ester, amide, tetrazole, SO_2NH_2), (v) substituents α to the CO_2H group (Me, Et), (vi) variation of adamantyl moiety (2-adamantyl, cyclohexyl, norbornyl, cyclopentyl, cyclohexenyl.

BIOLOGY

Pharmacology: 41 is not antiinflammatory in the sense of steroidal or conventional antiinflammatory drugs. It did not, for instance, protect against experimental adjuvant arthritis or the growth of cotton pellet granulomas. However, it did inhibit the early inflammatory response, reported to be histamine mediated, to paw-injected carrageenin without preventing the later phase of swelling. It was this response which was used as a primary screen for further development of analogs. None of the above variants was sufficiently active for further pursuit as an antiallergic drug except for the compound in which the carboxyl group of 41 was replaced by a sulfonamide group. This compound was 0.5 x 41 in the canine asthma assay, of which mention will be made shortly.

41 was studied in a variety of test systems, both in vivo and in vitro, and had antiallergic properties apparently dependent on the inhibition of cellular release of allergic mediators from cells. More specifically: (i) in the guinea pig anaphylaxis model, pretreatment with 41 inhibited the reaction by 69% at 100 mg/kg/p.o. It is of relevance that 41 given after anaphylaxis had occurred, inhibited 1 hour later the residual bronchoconstriction which clinically results in delayed deaths (Collier, 1967). Antihistamines do not inhibit this residual bronchoconstriction, indicating that 41 is capable of inhibiting mediators of this reaction which are insensitive to antihistamines, e.g., possibly SRS-A. In the canine asthma model (Oronsky, 1972), 41 given prophylactically (100 mg/kg/p.o.) was as effective as theophylline (70% inhibition at 100 mg/kg/p.o.). When administered i.v. at 40 mg/kg after the induction of an asthmatic attack (therapeutic), 41 gave 57% inhibition, whereas DSCG (3-5 mg/kg) did not inhibit the reaction. Finally, when mixed with the inducing antigen (inhalation) at 3 mg/kg, 41 inhibited the reaction 66%, whereas DSCG at the same dose inhibited 55%. 41 inhibited the dermal allergic reaction of sensitized Rhesus monkeys to Ascaris antigen in a dose-related inhibition at 30-125 mg/kg/p.o. Theophylline inhibited the monkey Ascaris skin allergy 90% at 12 mg/kg, whereas the antihistamine pyrilamine was ineffective at 10 mg/kg. 41 inhibited the allergic release of histamine by 80% at 100 mg/kg/p.o. from the rat peritoneal cavity. Comparable results have been obtained with DSCG (Orange, 1970). It was concluded that this profile of effects is consistent with the hypothesis that 41 would be useful in immediate hypersensitivity reactions due to release of allergic mediators. 41 was screened for endocrinologic, cardiovascular, neuropharmacologic and behavioral activities at effective antiallergic doses and was found to be devoid of activity.

Toxicology. In the form of a micronized suspension, the oral LD_{50} of 41 in rats was 1310 mg/kg and >4000 mg/kg if unmicronized. In mice, the oral LD_{50} of micronized suspension was 2000-2810 mg/kg and >4000 mg/kg in rabbits and >2000 mg/kg in dogs. When administered in various dosage forms for 14 days to mice and rats, large doses were tolerated without evidence of any notable specific pathology. Significant lymphopenia was noted at the highest dose (1000 mg/kg/p.o./day). Aside from occasional emesis and slight body weight loss, dogs gave no demonstrable response to doses of micronized 41 in capsules at doses of 25, 125, 750 mg/kg/p.o./day for 14 or 30 days.

Ulcerogenicity. No gastric ulcers or erosions were produced when either 300 or 600 mg/kg/p.o. of 41 were given orally to rats for 4 consecutive days.

Drug Metabolism. Plasma level time curves following oral administration of 41 indicate good absorption. Peak plasma levels (11.6 μg/ml) were observed within 30 min. after oral (20 mg/kg) administration of 41 to the rat. Dogs exhibited peak levels (20 μg/ml) within 2 hours of oral administration and man showed peak levels (10.5, 15.3 μg/ml) within 2-3 hours following administration of a tablet formulation of 41 at a dose of 10 and 20 mg/kg/p.o. respectively. 41 disappeared from the plasma of rats, dogs and man in a biphasic fashion. The initial half-life was 1-1.5 hours in both rat and dog, and 0.5-1 hour in man. The slower half-life was approximately 5.5 hours in rats, 2.4-6.6 hours in dogs and 8-12 hours in man. Administration of ten doses of 41 (20 mg/kg/p.o.) to the rat and dog did not appear to alter the time of peak drug levels or the rapid initial half-lives.

Clinical. 41 was well tolerated in single oral doses up to 1500 mg and in multiple doses up to 1500 mg q.i.d. for 28 days. In a double blind, placebo controlled (28 day) trial in patients with seasonal allergic asthma,(doses administered from 2-4 g q.i.d.) no apparent significant therapeutic differences in the many parameters studied were observed between the drug and placebo groups. In a final study, patients

with ragweed sensitive asthma in remission were given control observations which were repeated weekly on medication with 41. In no patients were the measured effects of bronchoprovocation or skin testing altered by treatment with the drug. Clinical studies were discontinued in 1975.

CONCLUSION

To date, the story of the development of antiallergic drugs, which have as their mechanism of action the inhibition of mediator release, has been a frustrating and disappointing one. However, as Francis Bacon wrote, "They are ill-discoverers who think that there is no land when they can see nothing but sea." In this respect antiallergy research seems at a crossroads. The PCA approach has been pursued vigorously with but limited clinical success. Research in this area was at an all-time high in 1979 and the number of publications has increased over the past year, despite doubts arising as to the predictive value of the PCA screen. An in-depth description of drugs affecting the respiratory system has been published (Temple, 1980), and now, with new knowledge of SRS-A and lipoxygenase inhibitors, in time a new generation of anti-allergy drugs will be found. In the meantime, the original goal, an orally active DSCG with good clinical efficacy, is still a viable one which hopefully will be achieved in the next few years.

Acknowledgments. I would like to express my appreciation to my coworkers in the story of GPA 2476. This reflects the work of Mr. S. Lopoukhine for the Chemistry, Drs. Perper, Oronsky, and Messrs. V. Blancusi and S. Macpherson for the Pharmacological Programme, Drs. E. Redalieu and G. Rodgers for the Drug Metabolism studies, Dr. R. Katz for the toxicology and Dr. W. Wagner for the clinical studies.

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Present Knowledge of GABA Receptor Agonists

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ABSTRACT

GABA receptor agonists affect several functions in the central nervous system.
1) Increase in cellular excitability leading to seizures is diminished whether it is induced by reduction in GABAergic transmission or by mechanisms which are primarily unrelated to GABA-mediated events. This predicts a therapeutic action of GABA receptor agonists in various forms of epilepsy.
2) Sedation and muscle relaxation are induced by doses of GABA receptor agonists by far higher than those which antagonize convulsions suggesting a low incidence of these side-effects in man.
3) Myorelaxation appears to be more marked in dystonia suggesting that this effect of GABA receptor agonists depends on the basal muscle tone.
4) Reduction by GABA mimetics in striatal DA turnover, together with their influence on striatal function via an unknown mechanism, suggests that increase in GABAergic transmission ameliorates L-DOPA-induced involuntary movements, neuroleptic-induced tardive dyskinesias and possibly some symptoms of Huntington's chorea.
5) Limbic DA turnover is only scarcely decreased by GABA receptor agonists, which might explain the failure of these agents to affect schizophrenic symptoms. Limbic NA turnover is, in contrast, enhanced by GABAergic drugs but the implication is unknown as no clear-cut clinical data are available on the effect of these agents on mood.
6) Other actions of GABA receptor agonists include decrease in striatal ACh and serotonin turnover, but their implications in human central nervous system disorders are still speculative.

KEYWORDS

GABA receptor agonists, muscimol, progabide, dopamine, acetylcholine, serotonin, noradrenaline, GABA, convulsions, dystonia, extrapyramidal disorders, limbic disorders

1. INTRODUCTION

Alterations of γ-aminobutyric acid (GABA)-mediated transmission are probably involved in neurological disorders such as Parkinson's disease, Huntington's chorea and epilepsy. Moreover, changes in GABA synaptic activity may be implicated in iatrogenic syndromes including L-DOPA-induced involuntary movements and tardive dyskinesias caused by neuroleptic agents. In some of these disorders a (relative) decrease

in GABA-mediated events seems to be a pathogenetic factor (see below). On this basis, compounds which increase GABAergic transmission might have a therapeutic potential. However, with only a few exceptions, the available GABAergic agents possess several drawbacks (see below) which have prevented a systematic investigation of their neuropharmacological, neurochemical and clinical spectra and, therefore, of the role of GABA in the central nervous system. In the following, the present knowledge of the effects of the enhancement of the GABAergic transmission by GABA receptor agonists will be reviewed briefly with emphasis on the two most extensively investigated compounds, muscimol and progabide.

2. GABA-TRANSAMINASE INHIBITORS - GABA RECEPTOR AGONISTS

Increase in GABAergic transmission can be effectively induced by 1) inhibitors of GABA-transaminase (the enzyme which degrades GABA) which enhance the synaptic concentration of the amino acid, or 2) stimulation of GABA receptors. In fact, GABA itself does not readily enter the brain and can not be considered a therapeutic agent (Tower, 1976).

Inhibitors of GABA transaminase such as aminooxiacetic acid, γ-vinylGABA, γ-acetylenic GABA, ethanolamine-O-sulphate, Na-valproate, have the drawback of inhibiting, to different extents, also the activity of glutamic acid decarboxylase, the enzyme which synthesizes GABA. Thus, in high doses, these compounds may cause seizures possibly due to a decrease in GABA levels leading to a reduction in GABAergic transmission. In addition, these agents, e.g. aminooxiacetic acid, are toxic or virtually do not cross the blood brain barrier (e.g. ethanolamine-O-sulphate) (cf. Bartholini, 1980).

Accordingly, a broad therapeutic potential of GABA transaminase inhibitors is, at the present, questionable. An exception is Na-valproate which displays a therapeutic action in epilepsy. This compound seems to block succinic dehydrogenase rather than GABA transaminase activity.

Among the most known and specific GABA receptor agonists, muscimol has a high affinity for GABA receptors but only scarcely penetrates into the brain, is rapidly metabolised and is toxic (Baraldi and others, 1979; Chase and Tamminga, 1979 ; Enna and others, 1980). THIP (Krogsgaard-Larsen and others, 1979) isoguvacine and kojic amine have been synthesized recently and the knowledge of their pharmacological and neurochemical profile is still fragmentary. Progabide {SL 76 002 ; 4-{[(4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)methylene]amino}-butanamide}* (Bartholini and others, 1979c ; Kaplan and others, 1980 ; Lloyd, Worms and others, 1979) is probably the GABA receptor agonist which has been most exhaustively investigated in experimental pharmacology and clinical research. Progabide, in the mammalian organism, penetrates into the brain and by deamination of the amide moiety and cleavage of the imine bond gives rise to its acidic metabolite SL 75 102 and to GABAMIDE, respectively, both of which, by similar transformations form GABA itself (Bartholini and others, 1979c ; Worms, Depoortere and others, in preparation).These metabolites are found in the central nervous system where they must have originates as both GABAMIDE and GABA virtually do not cross the blood brain barrier. Progabide displaces ^{3}H-GABA from binding sites of brain membrane preparations (rat and human)(Bartholini and others, 1979c) ; the compound diminishes the firing rate of dorsal Deiter's nucleus neurons (which receive a major GABAergic input from cerebellar Purkinje cells) and antagonizes the increase of their firing caused by blockade of GABAergic transmission by picrotoxin (Worms, Depoortere and others, in preparation). Similarly, SL 75 102 and GABAMIDE (and obviously GABA) display GABA receptor agonist properties. Thus, both compounds displace labelled ligands from GABA receptors (Bartholini and others, 1979c) ; SL 75 102 has an effect identical to that of GABA (on- and off-times ; cross desensitization ; reversal potential) in depolarizing, by a bicuculline and picrotoxin sensitive

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*Synthesized by Dr. J.P. Kaplan, Chemistry Department, Synthélabo(L.E.R.S.), Paris.

mechanism, rat dorsal cells "in vitro" (Desarmenien and others, 1980). Furthermore, both progabide and SL 75 102 are inactive in displacing ligands for dopamine, serotonin, glycine, histamine, opiate, α- or β-adrenergic receptors (Lloyd, Arbilla and others, in preparation) and virtually do not affect L-glutamic acid decarboxylase and GABA transaminase activity or GABA uptake (Bartholini and others, 1979c ; Lloyd, Arbilla and others, in preparation). Accordingly, progabide has to be considered a pure GABA receptor agonist and a precursor of GABA receptor stimulants including GABA itself.
Other compounds affecting GABA-mediated events such as GABAculine and hydrazine propionic acid, (GABA-transaminase inhibitors) or trans-4-amino-crotonic acid and aminopropane-sulfonic acid (GABA receptor agonists) have also been only scarcely investigated and will not be dealt with here.

3. ACTIONS AND SITES OF ACTIONS OF GABA RECEPTOR AGONISTS IN THE CENTRAL NERVOUS SYSTEM

3.1. Cellular Excitability .

GABA receptor stimulants antagonize the increase in cellular excitability induced by various mechanisms (Worms, Depoortere and others, in preparation ; Lloyd, Worms and others, 1979). Thus, apart from the antagonism of progabide to the picrotoxin-induced increase in firing rate of Deiter's nucleus neurons (see above), muscimol and progabide prevent both electrical and behavioural manifestations of seizures linked to blockade of GABAergic transmission by bicuculline or picrotoxin in the rat. Also, the compounds block the focal epilepsy induced by cortical injections of penicillin in the cat - as well as metrazol convulsions in the rats - models which probably involve GABA-mediated events. Muscimol and progabide appear to be the most active compounds (apart from benzodiazepines) on the majority of seizure models ; but, of particular interest, is the fact that the two drugs also prevent convulsions caused by mechanisms which do not seem to be primarily connected with GABA-mediated transmission. These include strychnine- and electroshock-induced seizures as well as audiogenic seizures. This property is shown by other GABAergic compounds, such as Na-valproate. In contrast, commonly used anticonvulsant agents such as diphenylhydantoin, or ethosuccimide are active only in a few of the test models described above (Worms, Depoortere and others, in preparation ; Lloyd, Worms and others, 1979).
The interest of GABAergic agents also lies in the fact that their therapeutic ratio -acute lethality (LD_{50}) : ED_{50} in anticonvulsant models - is higher than that of diphenylhydantoin and ethosuccimide (Worms, Depoorter and others, in preparation ; Lloyd, Worms and others, 1979). Also, the ratio of sedation (as measured by locomotor activity) or myorelaxation (loaded grid test) : average ED_{50} for anticonvulsions, is higher than that of commonly used antiepileptic agents (Worms, Depoortere and others, in preparation ; Lloyd, Worms and others, 1979).The incidence of these side-effects differentiates GABA receptor agonists from benzodiazepines, which have ED_{50}'s values in anticonvulsant tests close to the doses which induce sedation and myorelaxation (Worms, Depoortere and others, in preparation ; Lloyd, Worms and others, 1979).
In conclusion, increase in GABAergic transmission 1) allows a control of convulsions which involve a reduction of, or which are independent from primary changes in, GABA activity ; 2) when elicited by anticonvulsant doses of pure GABA receptor agonists, causes only slight sedation and muscle relaxation.

3.2. Extrapyramidal System .

3.2.1. Dopamine neurons. Although few reports have indicated that (in particular experimental conditions) GABA might (indirectly) stimulate the activity of nigrostriatal dopaminergic neurons (Cheramy, Nieoullon and Glowinski, 1978 ; Dray, 1979 ; Grace and Bunney, 1979), a great deal of data points to an inhibition of this pathway by GABA receptor agonists. Thus, progabide and muscimol diminish in the rat the

synthesis of DA as estimated from the reduction of DOPA accumulation following inhibition of DOPA decarboxylase "in vivo" and the formation of $^{14}CO_2$ from 1-^{14}C-tyrosine in striatal slices. This latter effect is completely antagonized by picrotoxin. Also, GABA receptor agonists reduce DA utilization as they retard the α-methyl-p-tyrosine (αMT)-induced striatal DA disappearance. In addition, progabide diminishes the basal output of DA in the cat caudate nucleus perfused by means of the push pull cannula. These effects of progabide on DA synthesis and release occur at threshold doses of 100-200 mg/kg ip and are significantly more pronounced after neuroleptic agents (αMT, DOPA synthesis, tyrosine hydroxylase activity, push pull cannula experiments). This suggests that DA neurons are under a state of tonic inhibition and only after activation (e.g. by neuroleptics) become more sensitive to increase in GABAergic transmission (Bartholini and others, 1979c ; Scatton, Zivkovic and others, in preparation). Behavioural results are in line with the decrease in DA turnover by GABA receptor agonists. Thus, although these compounds, per se, do not induce catalepsy, they markedly potentiate the cataleptogenic action of neuroleptic agents (Worms and Lloyd, 1980 ; Worms, Depoortere and others, in preparation). This effect is explained by the reduction in DA neuron activity, DA release and DA availability at the receptor sites for competing with the neuroleptics.
In conclusion, GABA receptor agonists reduce DA turnover in extrapyramidal system and, therefore, DAergic transmission. This action suggests a therapeutic potential of GABA receptor agonists in extrapyramidal disorders (see section 4).

3.2.2. Cholinergic neurons. Cholinergic neurons in the striatum play a key role in modulating extrapyramidal function (Bartholini and Stadler, 1976) as they are tonically inhibited by nigro-striatal DA neurons and translate (in part) the changes in DAergic transmission into motor patterns. Thus, decrease in DAergic transmission (Parkinson's disease, neuroleptic-induced parkinsonism) enhances acetylcholine (ACh) turnover and results in extrapyramidal symptoms such as rigidity and tremor. On the contrary, increase in DA synaptic activity leads to reduction in cholinergic transmission and dyskinetic syndromes (DOPA-induced involuntary movements, neuroleptic-induced tardive dyskinesias, Huntington's chorea ; see section 4).
As increase in GABAergic transmission reduces DA neuron activity, it has to be expected that GABA receptor agonists accelerate ACh turnover in the striatum. On the contrary, compounds such as progabide and muscimol reduce the activity of striatal cholinergic neurons (Scatton and Bartholini, 1980a ; 1980b). Thus, these compounds - in the absence of changes in ACh-esterase or choline acetylase activity 1) increase the striatal ACh concentration (which reflects a decreased utilization) ; 2) retard the hemicholinium-3-induced ACh disappearance ; 3) reduce the synthesis of ACh from pyruvate in striatal slices (Scatton and Bartholini, 1980a ; 1980b). The reduction by GABA mimetics of ACh turnover occurs at doses by far lower than those which diminish DA neuron activity (e.g. threshold doses of progabide, 10-15 vs 100-200 mg/kg ip, respectively (Scatton and Bartholini, 1980b ; 1980c). This indicates that cholinergic neurons are more sensitive to GABAergic inhibition.
The inhibition of striatal ACh turnover has behavioural correlates. Thus, low doses of GABA mimetics 1) antagonize the cataleptogenic action of neuroleptics and 2) potentiate the stereotyped behaviour caused by DA receptor agonists. Both effects are likely the result of the reduction in ACh turnover by GABA receptor stimulants (see section 3.2.2.) (Worms and Lloyd, 1980 ; Worms, Depoortere and others, in preparation).
In conclusion, it appears that GABAergic agents diminish at low dose range ACh and at a higher range, both ACh and DA, turnover, the action of higher doses on DA being predominant.

3.2.3. Unknown neurons involved in extrapyramidal function. GABA receptor agonists affect striatal function also by an action independent from known neurons (Bartholini, Scatton and Zivkovic, 1980). This view is based on two sets of data. Firstly, in the high dose range, these agents potentiate the stereotyped behaviour induced by DA receptor stimulants, an effect which is independent from the activity of DA neurons and, therefore, from the reduction in DA release induced by GABA mimetics. Also, the

reduction in ACh turnover caused by GABA mimetics is excluded as it should lead to the opposite effect, namely, potentiation of stereotypies (see section 3.2.2.). Secondly, co-administration of progabide and neuroleptics for several days results in a reduction of the supersensitivity of DA target cells to DA receptor agonists, which develops during repeated treatment with neuroleptic agents.As this effect occurs without changes in the tolerance which DA and ACh neurons develop to repeatedly administered GABA mimetics or neuroleptics, it is likely that GABA receptor agonists act beyond both DA and ACh synapses (Bartholini, Scatton and Zivkovic, 1980) ; the site of action is, however, unknown.
In conclusion, the fact that GABA receptor agonists potentiate the stereotypies induced by DA receptor stimulants and prevent the supersensitivity caused by neuroleptics, suggests that increase in GABAergic transmission affects extrapyramidal function via unknown neurons involved in the functional output of the striatum. The bearing of this action on the treatment of extrapyramidal disorders will be dealt with in section 4.2.

3.3. Limbic System.

3.3.1. DA neurons. The turnover of DA in the mesolimbic pathway is reduced by progabide and muscimol (αMT, DOPA synthesis, tyrosine hydroxylase activity experiments) (Bartholini and others, 1979c). However, the doses of GABA receptor agonists which decrease limbic DA turnover are 3-5 times higher than those which affect extrapyramidal DA. Thus, limbic DA neurons are by far less sensitive to GABAergic inhibition than the nigrostriatal cells. The limited response of mesolimbic DA neurons might have some bearing on the treatment of schizophrenia with GABA receptor agonists (see section 4.3.1.).

3.3.2. Noradrenaline neurons. GABA receptor agonists enhance the turnover of noradrenaline (NA) in limbic areas (and hypothalamus) (Bartholini and others, 1979c ; Scatton, Dedek and others, in preparation). Thus, progabide or muscimol increase 1) the αMT-induced NA disappearance - an effect which is antagonized by picrotoxin - and 2) the levels of MOPEG-sulphate. The acceleration of NA turnover by GABA receptor stimulants is probably not the result of a direct action of these compounds on NA neurons as microiontophoretic application of GABA on locus coerulens inhibits the firing of NA cells (Guyenet and Aghajanian, 1979). Activation of these neurons must, therefore, result via an action on other, possibly serotonergic, neurons. Thus, GABAergic agents reduce serotonin neuron activity (see section 3.4.) which is known to inhibit NA cells.

3.4. Serotonin neurons.

GABAergic agents reduce in the rat striatum the α-propyldopacetamide-induced decrease in serotonin disappearance and the accumulation of the transmitter caused by pargyline (Scatton, Dedek and others, in preparation). This clearly indicates that the turnover of serotonin is decreased rather than increased as previously reported on the basis of the enhancement of tryptophane and 5-hydroxyindoleacetic acid levels (Bartholini and others, 1979c ; Biswas and Carlsson, 1977). Thus, the increase in brain tryptophane - which usually accompanies an enhancement in serotonin turnover - is the result of the displacement of the amino acid from serum proteins by GABA mimetics (Scatton, Dedek and others, in preparation). Also, the increase in cerebral concentration of 5-hydroxyindoleacetic acid - which might reflect an enhanced transmitter utilization - must rather result from an intraneuronal serotonin breakdown (Scatton, Dedek and others, in preparation). The decrease in serotonin turnover does not occur in limbic areas although they possess a serotonergic innervation. The reason is still unknown (cf. Scatton, Dedek and others, in preparation).

4. THERAPEUTICAL IMPLICATIONS

4.1. Epilepsy. Dystonia.

GABA receptor stimulants antagonize seizures caused in animals by various mechanisms. Thus, not only convulsions related to decrease in GABAergic transmission (a model of "petit mal") are affected by these agents but also those which do not seem to be primarily connected with changes in GABA-mediated events (see section 3.1.) (Worms, Depoortere and others, in preparation ; Lloyd, Worms and others, 1979). This allows the prediction that pure GABA mimetics will be therapeutically useful in human convulsions of various origins.
Also, the low incidence of sedation and muscle relaxation in animals predicts that GABA receptor agonists will cause a low incidence of these side-effects in man (Worms, Depoortere and others, in preparation ; Lloyd, Worms and others, 1979). Indeed, pilot trials in various, severe epilepsy forms have shown a marked therapeutic action of progabide, virtually in the absence of side-effects including sedation and myorelaxation. In contrast, the compound causes remarkable muscle relaxation in dystonic patients (Morselli and others, 1980). This might indicate that the muscle relaxant action of GABAergic agents depends on the basal muscle tone.

4.2. Extrapyramidal system disorders.

4.2.1. L-DOPA-induced involuntary movements. In Parkinsonian patients, L-DOPA causes involuntary movements which are due to a relative exaggeration of DA-mediated events. This is probably the result of an excessive formation and liberation of DA in the remaining DA neurons and/or of rapid changes in sensitivity of DA target cells (cf. Bartholini and others, 1979b). GABA receptor agonists by reducing DA neuron activity (see section 3.2.1.) might modulate DAergic transmission and reduce the involuntary movements. Also, the antagonism of GABA mimetics to the stereotypic behaviour induced by DA receptor stimulants (see section 3.2.3) suggests a therapeutic potential of GABAergic medication in this syndrome. Indeed, preliminary clinical trials have shown that progabide causes a noticeable amelioration of patients suffering from L-DOPA-induced involuntary movements (Bartholini and others, 1979b).

4.2.2. Huntington's chorea. Some symptoms of this disease (dyskinesias) are probably due to a (relative) exaggeration of DAergic transmission which may be linked to reduction of inhibitory (possibly GABAergic) input on DA neurons. Accordingly, diminution of DA neuron activity by GABAergic agents (see section 3.2.1.) might result in an amelioration. Preliminary clinical trials have shown some beneficial action of these agents, however, the results appear to vary from patient to patient, probably due to the different degree of brain degeneration (Morselli and others, 1980).

4.2.3. Neuroleptic-induced tardive dyskinesias. Repeated administration of neuroleptic agents to animals leads to supersensitivity of DA target system to DA receptor agonists (increased stereotyped behaviour) (for references, cf. Bartholini, Scatton and Zivkovic, 1980). It is, therefore, assumed that dyskinesias which occur in schizophrenic patients under neuroleptics result from a relative increase in DAergic transmission due to the development of supersensitivity to DA. GABAergic compounds will probably display a profilactic and a therapeutic action as they 1) decrease DA neuron activity, 2) prevent the supersensitivity caused by neuroleptics and 3) block the stereotyped behaviour induced by DA receptor stimulants (Scatton, Zivkovic and others, in preparation ; Bartholini, Scatton and Zivkovic, 1980) (see section 3.2.3.). Indeed, clinical results with progabide (Morselli and others, 1980) or valproate (Linnoila, Vinkari and Hietala, 1976) show a therapeutic action of these agents in neuroleptic-induced tardive dyskinesias.

4.2.4. Other indications. The reduction by GABA receptor agonists of striatal ACh neuron activity (see section 3.2.2.) suggests that low doses of these agents may be effective in Parkinson disease or neuroleptic-induced parkinsonism, disorders in which an increase in ACh turnover seems to play a major role. However, no clinical data are available as yet. Also, the implication of the reduction in serotonin turnover observed in striatum by GABA mimetics is still unknown.

4.3. Limbic system disorders.

4.3.1. Schizophrenia. Some schizophrenic symptoms are thought to depend on a relative increase in DAergic transmission in the limbic system (for references cf. Bartholini and others, 1979a). On this basis it can be assumed that reduction of DA neuron activity by GABA receptor stimulants is of therapeutic value in schizophrenia. However, clinical results with progabide (Morselli and others, 1980) and muscimol (Chase and Tamminga, 1979) have shown that these agents are therapeutically inactive in the psychosis. This may be due to the fact that mesolimbic DA neurons are only slightly sensitive to GABAergic inhibition (see section 3.3.1.).

4.3.2. Mood disorders. GABAergic agents accelerate the turnover of limbic NA (see section 3.3.2.) which appears to be involved in mood disorders. However, no clear-cut clinical data with these compounds have yet been reported although prevention of manic states seems to occur under valproate (Emrich, 1980).
Table 1 depicts sites of action and indications of GABA receptor agonists.

TABLE 1 Sites of Action and Indications of GABA Receptor Agonists

SITE OF ACTION	ACTION	THEORETICAL INDICATION	CONFIRMATION
Nigro-striatal DA neurons	↘	• Neuroleptic-induced tardive dyskinesias	yes
		• L-DOPA-induced involuntary movements	yes
		• Huntington's chorea	yes/no
Mesolimbic DA neurons	↘	• Schizophrenia	no
Striatal ACh neurons	↘	• Parkinson's disease • Neuroleptic-induced parkinsonism	?
Unknown system(s) distal to extrapyramidal DA and ACh neurons	?	• neuroleptic-induced tardive dyskinesias	yes
NA neurons	↗	• Mood	?
5-HT neurons	↘		
	Reduction of cellular excitability	• Seizures	yes
Cerebellum, spinal cord, (striatal muscles ?)	Reduction in muscle tone	• Dystonia	yes

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New Trends in Minor Tranquilizers

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ABSTRACT

The field of anxiolytic drugs is briefly reviewed. 1,4-benzodiazepines are recognized as the most useful drugs in treating anxiety of diverse origin. Significant advances in this class of substances are discussed and interesting representatives shown. Recent discoveries on the mechanism of action of 1,4-benzodiazepines have renewed and increased scientific interest in the whole field. New perspectives open up for the medicinal chemist searching for new anxiolytics.

KEYWORDS

Minor tranquilizer; anxiolytic; benzodiazepine; benzodiazepine binding site; benzodiazepine receptor; photoaffinity labelling; sleep inducer.

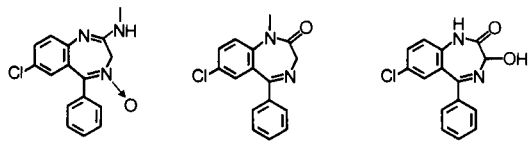
INTRODUCTION

The term "minor tranquilizer" has been used in the past in order to differentiate this type of drugs useful in treating the milder neurotic disturbances, from the major tranquilizers which were already established drugs in the treatment of psychotic disorders. Today, it seems more appropriate to call them anti-anxiety or better anxiolytic drugs. Anxiety and fear play a vital role in all human societies. To feel anxious in the face of a threatening stimulus is both normal and appropriate; it is only when the anxiety becomes so severe as to be incapacitating, or when it arises without reasonable cause, that clinical intervention is indicated. Unfortunately this occurs all too frequently. When we look back in history, we notice that mankind has probably always felt the need for alleviating anxiety and fear. The use and abuse of natural drugs like cannabis, opiates and alcohol which goes back to the dawn of our civilisation, may also be seen in this connection, but of course we cannot consider alcohol and cannabis to be the "first anxiolytics"! The proper treatment of pathological states of excessive anxiety and of deriving psychosomatic manifestations had to await a new milestone in psychopharmacology, the introduction of meprobamate into therapy in 1954. Previous treatments consisted in the administration of sedatives, mainly barbiturates which, although efficacious in small dosage, exposed the patients to the danger of overdose and addiction. Meprobamate resulted from an extensive effort to improve the short acting anxiolytic mephenesine, a sedative and muscle relaxant drug and it may

be considered the first proper anxiolytic drug. It became very popular, since it allowed the control of mild to moderate anxiety and tension in patients with neurosis or depression without causing too much sedation. Like all modern anxiolytics, it was also used and perhaps misused by normal individuals in order to overcome environmental or occupational stress situations. The success of meprobamate stimulated many laboratories to search for further improved anxiolytics. While many kept varying the propanediol and reserpine structures, the breakthrough happened to come from a broad screening program at Roche Nutley. As can be read in the benzodiazepine story by the chemist Sternbach (1978, 1979), a combination of serendipity and medicino-chemical intuition on his part and of organized pharmacological screening and astute observation on the part of the biologist Randall were the determinant factors in the discovery of chlordiazepoxide, 1957. With its unique success, chlordiazepoxide paved the way to a whole new series of therapeutically useful compounds: the benzodiazepines, which deserve a long chapter of their own.

1,4-BENZODIAZEPINES AND SIMILAR STRUCTURES

Although the name benzodiazepine has become synonymous of anxiolytic, the widespread use of benzodiazepine is due also to other qualities, shared in varying proportions by all members of the series, like their anticonvulsant, muscle relaxant and hypnotic (better sleep inducing) actions, combined with an extremely good tolerance. All these actions can be exploited therapeutically by the administration of adequate doses. I shall include these and other properties in my presentation, but of course I shall not be able to give a complete account of the many compounds and their manyfold aspects. I shall try to illustrate by some arbitrarily selected examples how the field evolved and which might be the trends. The prototype chlordiazepoxide reached the market in 1960. Sternbach and his group quickly found that the amidino group and the N-oxide were unnecessary ornaments. Essential was the 7-membered lactam as present in diazepam. Diazepam is more potent than chlordiazepoxide and proves very versatile in therapy. Since its marketing in 1963, it had an unprecedented success. The next compound to reach the market was oxazepam in 1965, developed by Wyeth; it is a metabolite of both, diazepam and chlordiazepoxide.

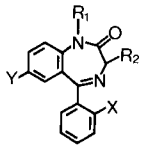


Solubility in H_2O, mg/ml	2	0.05	0.03
P n-Octanol-buffer, pH = 7.4	295	630	174
pKa	4.76	3.17	1.62

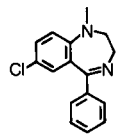
Fig. 1. First anxiolytics from the class of 1,4-benzodiazepines.

The many variations of the diazepam molecule which followed, quickly showed that the only changes allowed were in the positions 1, 3, 7 and 2' (Sternbach, 1964, 1973). Nevertheless, thousands of compounds were synthesized all over the world and numerous were found active. The isosteric thieno- and pyrazolo-1,4-diazepinones represented by the anxiolytics clotiazepam (Nakanishi, 1972; Nakazawa, 1975) and ripazepam (DeWald, 1973) are in fact so close geometrically and biologically to the benzodiazepines that they may be also classified amongst the classical compounds. On the whole, we count

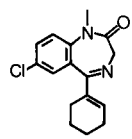
about 20 benzodiazepines of the classical type on the world market. Although some have grown to a respectable size, none has yet beaten the polyvalent diazepam.



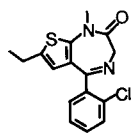
		R_1	R_2	Y	X
Nitrazepam	1965	H	H	NO_2	H
Temazepam	1970	Me	OH	Cl	H
Clorazepate	1972	H	COOK	Cl	H
Desmethyldiazepam	1973	H	H	Cl	H
Flunitrazepam	1975	Me	H	NO_2	F
Pinazepam	1975	$CH_2C{\equiv}CH$	H	Cl	H
Lorazepam	1977	H	OH	Cl	Cl
Prazepam	1977	CH_2–cyclopropyl	H	Cl	H
Nimetazepam	1977	Me	H	NO_2	H



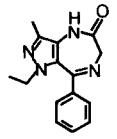
Medazepam
1968



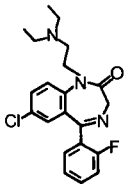
Tetrazepam
1974



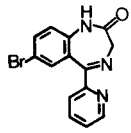
Clotiazepam
1979



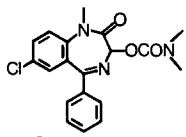
Ripazepam



Flurazepam
1970



Bromazepam
1974



Camazepam
1977

Fig. 2. Examples of marketed drugs from the class of 1,4-benzodiazepines.

Pharmacology

The 4 prominent pharmacological properties of e.g. diazepam are the anticonflict activity in rats and monkeys, the anticonvulsant activity in mice, the muscle relaxant activity in mice and cats, the sedative and sleep inducing activity in mice and rabbits.

TABLE 1 Pharmacological Profile

	Chlordiazepoxide	Diazepam	Oxazepam
Rat Conflict (see: Cook, 1973), MED p.o. mg/kg	2.2	0.63	1.25
Monkey Conflict MED p.o. mg/kg	0.6	0.31	
Mouse a-pentetrazole (see: Blum, 1973) ED 50 p.o. mg/kg	13.2	2.2	8.4
Cat relaxation (see: Randall, 1974) MED p.o. mg/kg	2.0	0.2	1.0
Barbital potentiation ED 50 p.o. mg/kg	8.1	1.5	0.44
Motor activity decrease to 40% p.o. mg/kg	42.0	29.0	27.0
3H-diazepam binding IC 50 nM/l	350.0	8.1	17.7
Average clinical dose (see: Cook, 1973) p.o. mg/day	40.0	20.0	49.0

A very low toxicity and lack of peripheral side-effects complete the picture. In developing new compounds, it proved quite difficult to derive the highly desirable differentiation of the 4 main properties from the animal screening data. In case of the anxiolytic activity, which interests most, behavioral tests are considered to give the best measure. They are usually carried out with trained rats or squirrel monkeys and active anxiolytics produce a normalisation of behavioral responses, which

have been suppressed by punishment or absence of reward (Sepinwall, 1978). It is important to note that this effect, which may be termed in a more general manner "behavioral disinhibition" does not consist in a depression of behavior, but in a distinct increase of behavioral responses. The results obtained reflect a complex situation and require a careful interpretation with additional conventional testing. They allow to differentiate early benzodiazepines from other psychotropic drugs and to a certain degree also between themselves. In Fig. 3, we see results obtained in a rat conflict test with the new sleep inducing agent midazolam, to which I shall come back later, and the anxiolytic diazepam. In comparing the two compounds, we notice that both have the same M.E.D. which is a measure of potency, but that the further development of the curves of punished and unpunished responses differs markedly. The punished response curves reflect the intensity of behavioral disinhibition, while the curves of unpunished response correlate with the sedative effects. The range of doses at which behavioral disinhibition predominates is an important factor in assessing the anxiolytic properties of a new compound.

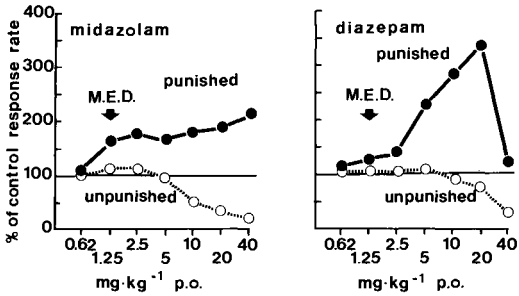


Fig. 3. Effect of midazolam and diazepam on punished and unpunished responses in a rat conflict test (Pieri, 1980).

The choice is influenced also by other criteria, e.g. the duration of action. In Fig. 4, the duration of action of the two compounds midazolam and diazepam is shown using a biochemical index, the levels of cyclic GMP in rat cerebellum, which reflects the behavioral stimulation. While a prolonged action is certainly wellcome for an anxiolytic like diazepam, the opposite is the case for a sleep inducer. Thus midazolam qualifies well for the latter indication.

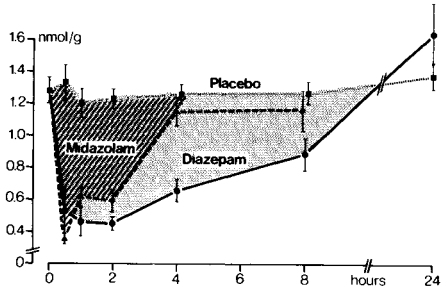


Fig. 4. Decrease of cyclic GMP content in rat cerebellum (Burkard, 1979).

This example should explain why already slight differences in the pharmacological profile have led to clinical trials of quite a number of benzodiazepines, some of which in the hands of the clinicians proved indeed to be especially suitable for

the treatment of particular diseases. Typical examples are the anticonvulsant clonazepam and the sleep inducers flurazepam, nitrazepam and flunitrazepam. More difficult was the improvement of the anxiolytic component, as achieved for example with bromazepam. A more specific muscle relaxant was not introduced yet, the indication muscle spasm and tetanus is still covered mainly by diazepam.

Other Biological Activities

Antipsychotic. Attempts have been made to use diazepam in high-dosage for the treatment of schizophrenic patients. Trials are under way to confirm the possible antipsychotic properties of diazepam (Beckmann, 1980).

Antialdosterone and diuretic. Ro 13-1870 has been selected from a large series of 7-ureido-1,4-benzodiazepines active in adrenalelectronized rats (Fischli, 1980), and submitted to clinical trials.

Schistosomicidal. Some 7-nitro-1,4-benzodiazepine-2-ons were found to be very active in vitro and in vivo (Szente, 1975; Stohler, 1978). Preliminary clinical trials with Ro 11-3128 confirm the activity in humans, but strong central effects seem to hinder a broad application in the therapy of schistosomiasis. The action of Ro 11-3128 on schistosomes is clearly mediated by receptors that are different from those involved in central nervous system effects and which we are going to discuss later. On the other hand, Ro 11-3128 is also very interesting as potent anxiolytic.

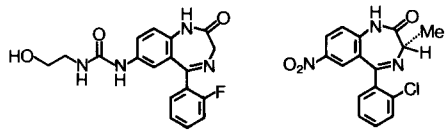
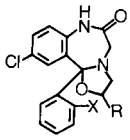


Fig. 5. 1,4-Benzodiazepines with different biological activities.

Chemical Variations

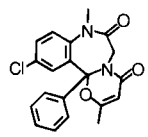
Anellation. Imaginative chemists have of course also tried major changes of the original structure. After some pitfalls, interesting trends emerged. Early and successful examples are the 4,5-anellations (Miyadera, 1971; Lemke, 1971; Derieg, 1971), which led to the drugs oxazolam, cloxazolam (Miyadera, 1971; Kamioka, 1972) and ketazolam (Szmuszkovicz, 1971). These compounds can be considered to be prodrugs. Studies in rats have shown that the additional ring may be removed metabolically (Yasumura, 1971). Of particular interest are anellations involving the 1,2-position as exemplified by the following triazolo derivatives: the sleep inducers estazolam (Meguro, 1970), triazolam (Hester, 1971) and the anxiolytic alprazolam (Hester, 1971). Isosteric analogs, thieno-triazolo-diazepines attracted also much interest and proved highly potent, e.g. Ro 11-7800 (Hellerbach, 1979), etizolam (Tahara, 1978) and brotizolam (Weber, 1978). The additional triazolo ring imparts new properties to the molecule, it increases affinity for the benzodiazepine receptor and potency in vivo, opens new metabolic pathways, e.g. by the easy hydroxylation of the 1-methyl group (if present) and stabilizes at the same time the diazepine ring against degradation. Triazolam, as an example, has become a successful sleep inducer active at extremely low doses (0.25 - 1 mg). Very interesting properties were discovered by the 1,2-anellation with the more basic imidazo ring, as demonstrated by the

already mentioned sleep inducer midazolam (Walser, 1978a) and the potential anxiolytic Ro 21-8384 (Walser, 1978b).

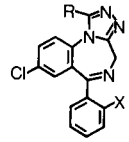


Oxazolam X = H R = Me
1971

Cloxazolam X = Cl R = H
1974



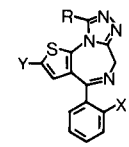
Ketazolam
1980



Estazolam R = H X = H
1975

Alprazolam R = Me X = H
1979

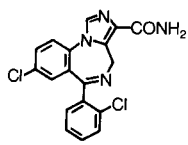
Triazolam R = Me X = Cl
1977



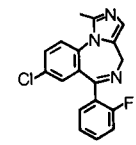
Ro 11-7800 R = CH_2NH_2 X = Y = Cl

Brotizolam R = Me X = Cl Y = Br

Etizolam R = Me X = Cl Y = Et



Ro 21-8384



Midazolam

Fig. 6. 4,5- and 1,2-anellated benzo- resp. thieno-1,4-diazepines.

Water soluble compounds. Due to the basic nitrogen in position 2 (pK_a = 6.15), midazolam salts have the advantage to form stable water solutions at a physiologically acceptable pH between 3 and 4, at which the diazepine ring is largely closed. This is a remarkable progress, since as yet, solvents were necessary in order to solubilize benzodiazepines for i.v. or i.m. application, for example as anticonvulsants or pre-anaesthetics. Although diazepam solutions in for example propyleneglycol-water are well accepted, chemists faced for a long time the wish of clinicians to have at their disposal a fully active but water soluble benzodiazepine derivative.
Midazolam has indeed demonstrated his utility as an extremely well tolerated injectable for premedication and induction of anaesthesia in ongoing clinical trials. The already mentioned Ro 11-7800 was also designed as water-soluble compound and carries therefore the 1-amino-methyl group. Unfortunately it showed insufficient longterm stability in solution. In this connection let me refer to just another interesting approach to the problem of obtaining aqueous injectable formulations. It makes use of a well-known phenomenon, the easy ring closure of o-glycylaminobenzophenones, also called open form of 1,4-benzodiazepines, under physiological conditions. It is known that this is a reversible process; at low pH, the 4,5-imine bond is quickly hydrolysed (Sternbach, 1963) and reformed again by raising the pH to neutrality. The rate of equilibrium is strongly influenced by the substituents in N-1 and the aromatic rings (Hassall, 1977). Water-soluble pro-drugs have been prepared by synthesizing peptidoaminobenzophenones. Through the action of peptidases, the latter release o-glycylaminobenzophenones, which quickly cyclise as mentioned above. The synthesis and properties of peptidoaminobenzophenones were described by researchers of Shionogi (Hirai, 1980) and Roche Welwyn (Hassall, 1977). The latter called their products appropriately "latentiated" 1,4-benzodiazepines. Ro 3-7355 for example, which is a pro-drug of diazepam, was studied in detail. In contrast to small animals, the peptidases in man did not work fast enough. The onset of activity was somewhat delayed and rendered the drug unsuitable for some of the emergency indications foreseen (for example epilepsy). Although claims have been made that some o-glycylaminobenzophenone derivatives are active per se and thus build a new class of anxiolytics, I think that in every case their activity can be explained by metabolic

formation of 1,4-benzodiazepines as exemplified above (see also Lahti, 1976).

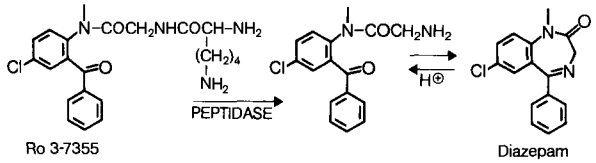


Fig. 7. Latentiated benzodiazepines.

Related Structures

5-Phenyl-1,5-benzodiazepinones. The presence of the imine group in 4,5 was believed to be essential for the activity of benzodiazepines (Gschwend, 1979). The isomeric 1,5-diazepines show on the contrary that active compounds may be generated without it. The planarity of the new lactam 4,5 bond may explain this fact, since it mimicks the imine group and maintains the same geometry of the 7-membered ring. While, in general, the same SAR apply for 1,5 and 1,4-benzodiazepines, it is interesting to note that the 1,2-anellation with 1-methyl-triazole did not give the expected rise in potency (Moffett, 1976). In spite of its rather low pharmacological potency (Barzaghi, 1973), clobazam (Rossi, 1969) was successfully introduced into therapeutic practice as anxiolytic with apparently little sedative side-effects (Radmayr, 1980).

1-Phenyl-2-benzazepines. Similar considerations about the importance of the shape of the 7-membered ring and the overall geometry may be applied to the 2-benzazepines, which are a major departure from the original benzodiazepine skeleton. The anellation in position 1,2 with a pyrimido ring leads to pharmacologically interesting compounds. Ro 22-3245 was selected for clinical trials as anxiolytic.

3,4-benzodiazepines. Tofisopam (Petőcz, 1971) bears a merely formal relationship with the 1,4-benzodiazepine, because of its chemical nomenclature! There is little structural and pharmacological similarity to be found, and it lacks of course affinity to the benzodiazepine receptor. It is therefore surprising and interesting that at doses of 100 - 150 mg/day, it has been reported clinically effective as anxiolytic (Goldberg, 1979).

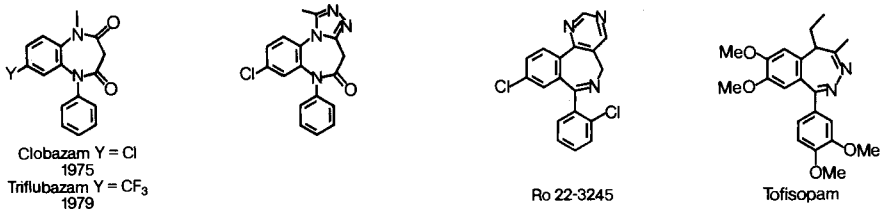


Fig. 8. Related Structures.

Mechanism of Action

For many years, benzodiazepines have eluded all attempts to explain their mode of action. With time, the view emerged clearly that benzodiazepines have a specific profile of activity differing from previously used agents like barbiturates and meprobamate. This view finds now a beautiful confirmation by the progress achieved in recent years. First came electrophysiological and pharmacological evidence for

the hypothesis that benzodiazepines influence the GABA system in the CNS (Haefely, 1975; Costa, 1975). GABA is the most widespread synaptic transmitter in the CNS, and operates in neurones that exert an inhibitory role. Benzodiazepines increase the GABA-energic synaptic transmission (Haefely, 1978) and thus achieve a depressant effect which may explain their anticonvulsant, muscle relaxant, sleep inducing and probably also their anxiolytic properties. It means that benzodiazepines act by enhancing a specific physiological inhibitory mechanism, which controls the normal function of the CNS. We should not overlook this fundamental and satisfactory conclusion, which amply justifies the great effort put by researchers and the involved industries into the benzodiazepine field. The specific mode of action of benzodiazepines is probably at the root of their therapeutic success. Recent in vitro experiments confirm again, now at a molecular level, the high specificity of action of the benzodiazepines. I am referring to the discovery of high affinity binding sites for benzodiazepines in membrane preparations from animal and human brain (Möhler, 1977; Squires, 1977). They are currently called benzodiazepine receptors (BR) and they possess all required qualities such as high affinity, stereospecificity, saturability, and they are sensitive to heat or proteolytic enzymes. The importance of this discovery was recognized also at this symposium by devoting a separate presentation on this subject (Garattini, 1980). For this reason, I shall limit my presentation and illustrate first of all the in vitro binding results expressed as IC 50 of many of the compounds I mentioned. These data were elaborated by Möhler in our laboratories in Basle. Looking at the 1,4-benzodiazepines, it is easy to distinguish the direct acting drugs from the pro-drugs, the latter requiring first a metabolic transformation to become active. This is the case of the prototype chlordiazepoxide, which has to be hydrolysed to the 1,2-lactam, of medazepam which must be oxidized in position 2, of oxazolam and cloxazolam, which must loose the extra 4,5-anellation and of the benzophenone derivative Ro 3-7355, which requires a peptidase in order to get ready for cyclisation to diazepam, as we already have discussed. The affinity of the 1,5-benzodiazepine clobazam is rather low for a direct action. Tofisopam is either acting by a completely different mechanism or by metabolic activation. On the other hand, 2-benzazepines, like Ro 22-3245, behave normally. Among the other structures, it is interesting to note that many classic anxiolytics of the old generation, like phenobarbital, meprobamate, benactyzine, benzoctamine as well as newer compounds, like brofoxine and fenobam, together with the β-blockers propranolol and practolol, have no affinity to the BR. There are interesting exceptions, which demonstrate that completely different structures may bind, and as shown by further in vivo testing, also exert an effect on the BR. We shall come back to them later. (See TABLE 2.) I leave now the binding data in order to say a few words about the microscopic localisation of the BR and ask in particular if we can unify the electrophysiological evidence, which indicates an interaction of the benzodiazepines with the GABA-ergic synaptic transmission, and the biochemical notion about the BR. Are the BR at least in part physically associated with the GABA-ergic synaptic contacts? The answer is given on the next two slides. They show electron microscopic pictures from a yet unpublished work by Möhler, Wu and Richards (1980a). (See Fig. 9.) Two different techniques had to be employed in order to visualize on one side the BR, on the other the GABA-ergic nerve terminals. In the first case, the autoradiography with a tritium labelled benzodiazepine, which for this purpose had to be irreversibly bound to the BR, was used. In the second case, the tissue sections were stained with antibodies against glutamic acid decarboxylase (GAD). The need to form an irreversible, say covalent, bond between a benzodiazepine and its receptor stems originally from research directed at the isolation of the BR and was not an easy task. I would like to mention just one attempt, the proposal of irazepine (Rice, 1979), a benzodiazepine carrying a reactive isothiocyanatoethyl group in position 1. Photoaffinity labelling is the elegant solution to the problem, and it is very fortunate that some of the benzodiazepines with the highest affinity to the BR were found to be ideal substrates for photolabelling, namely the 7-nitrobenzodiazepines (Battersby, 1979; Möhler, 1980b). In our case, 3H-flunitrazepam was injected i.v. to rats, the animals sacrificed and brain slices prepared, exposed to U. V.

light and fixed for autoradiography. The nature of the heat-, light- and detergent-stable linkage between the 3H-flunitrazepam and the BR is not known. It could involve the nitro-group itself, which activated by the irradiation would easily react with an amino, hydroxy or thiol group held in a favorable position by the receptor binding forces.

TABLE 2 IC 50 for in Vitro Inhibition of Specific 3H-Diazepam Binding to Synaptosomal Preparations (P 2) from Rat Cerebral Cortex (Möhler, 1977).

1,4-Benzodiazepines

Directly Acting

Diazepam	8.1	Chlorazepate	59.0	Bromazepam	18.0	Brotizolam	1.2
Oxazepam	17.7	Flunitrazepam	3.8	Triazolam	4.0	Ro 21-8384	3.8
Nitrazepam	9.9	Ro 11-3128	1.2	Midazolam	4.8	Estazolam	8.5
Flurazepam	17.6	Lorazepam	3.5	Ro 11-7800	2.9	Irazepin	30.0

Pro-Drugs

Chlordiazepoxide	352.0	Oxazolam	>1000	Ro 3-7355	420.0
Medazepam	870.0	Cloxazolam	>1000	Ro 13-1870	>1000

1,5-benzodiazepines		3,4-benzodiazepines		2-benzazepines	
Clobazam	130.0	Tofisopam	>1000	Ro 22-3245	2.8

Other Structures

Phenobarbital	>1000	Benzoctamine	>1000	Nabilone	>1000
Meprobamate	>1000	Hydroxyzine	>1000	Fenobam	>1000
Benactyzine	>1000	Propranolol	>1000	Cl 218872	81.0
Tetrabenazine	>1000	Practolol	>1000	Zopiclone	31.0
Benzquinamide	>1000	Brofoxine	>1000	Ethyl-β-carboline-3-carboxylate	0.8

Our slides beautifully demonstrate the close interrelation at least between some of the GABA-ergic synaptic contacts and the BR. The first shows the autoradiographic picture of the BR in a region of contact between nerve terminals, the second the BR in proximity of a GABA-ergic nerve terminal stained for GAD. The rapid progress achieved has increased enormously the scientific interest in benzodiazepines, and we can expect further improvements of our knowledge on their mechanism of action in the next future. The situation reminds us of that in the analgesic field, where binding studies culminated in the discovery of the endorphins and subsequently brought new insights into the whole field. This analogy has already stimulated many laboratories to search for an endogenous agent, which could either be an anxiolytic or an anxiogenic agent. However, when measured by their IC 50 in the 3H-diazepam binding assay, which ranges between 1 - 5 µM, nothing exciting has been found among the so far proposed, possible endogenous ligands. The compounds are: inosine, hypoxanthine (Skolnick, 1978; Asano, 1979) and nicotinamide (Möhler, 1979). The latter is the only one showing pharmacologically and electrophysiologically very weak benzodiazepine-like effects in vivo (Möhler, 1979). Also of interest is the recently published case of ethyl β-carboline-3-carboxylate, which originating from an attempt to isolate an endogenous ligand from human urine, is in reality an artifact. However, it possesses with an IC 50 of 0.8 nM/l a high affinity to the BR (Braestrup, 1980). Pharmacological effects were not yet described. This discovery demonstrates that structures other than 1,4-benzodiazepines may also strongly bind (interact?) with the BR. Much endeavour is directed at the isolation (Lang, 1979; Battersby, 1979; Yousuffi, 1979; Möhler, 1980b, 1980c) and at the differentiation (Squires, 1979; Klepner, 1979) of the BR. It is the latter direction, which, I think,

will interest and influence medicinal chemists most. A recent paper (Sieghart, 1980) shows clear cut evidence for at least 2 different classes of benzodiazepine binding sites.

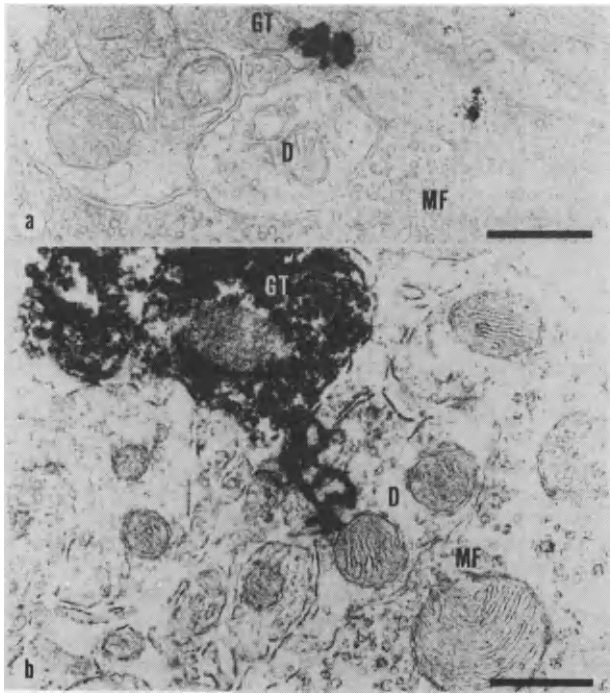


Fig. 9. Electron microscopic autoradiographs of BR in rat cerebellum. Photolabelling was performed after i.v. injection of 3H-flunitrazepam and fixation. Golgi nerve terminal (GT) adjacent to the dendrite (D), which is contacted by the mossy fiber (MF). The photolabelled nerve ending shows an immunocytochemical reaction with GAD antiserum. Bar = 0.5 µm (Möhler, 1980a).

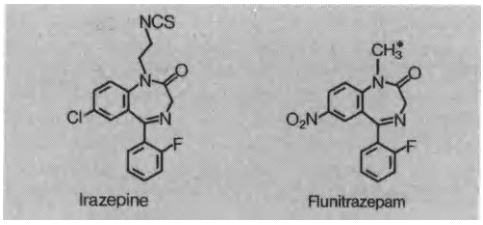


Fig. 10. Labels for the benzodiazepine receptor.

Using the above described photolabelling technique, which proves again its great usefulness, when followed by electrophoresis and fluorography, the authors were able to separate from rat brain membranes of different regions (for example hippocampus) two labelled protein fractions with molecular weights of $51 \cdot 10^3$ and $55 \cdot 10^3$. We can imagine that on this basis, drugs acting at different BR's could well be identified. Now that our knowledge of the BR's is rapidly growing and so many agonists are known,

one may ask whether there exist also antagonists. They would provide useful research tools and offer also a practical and desirable mean of cutting down the central effects of benzodiazepines, once not required any more, or undesired at all, like for example in anaesthesia and intoxications. By definition, an antagonist should possess affinity for the BR, displace benzodiazepines or other agonists, but fail to exert the normal effect. A look at the literature shows that little is known on this subject yet, but antagonists have been discovered very recently by our research group and details will be published in due course. The efforts to further clarify the mechanism of action of benzodiazepines will lead to an improved knowledge of fundamental biological processes in the CNS and will also open new opportunities for drug research, especially in the anxiolytic field. The following last chapter of my presentation is devoted to other anxiolytic structures and is probably one which will be most influenced by the new mechanistic insights. Medicinal chemists are already at work and certainly this subject will change and quickly grow in the next future.

Other Structures

β-blockers. Anxiolytic properties are ascribed to some β-blockers, which are widely used in stress situations. It remains doubtful whether their therapeutic effect is of central nature and not rather due exclusively to the inhibition of peripheral adrenergic β-receptors. There is clinical evidence that the peripheral effect is predominant with propranolol (Granville, 1966). Another example in this direction is the clinically active practolol, which has been shown to cross only very poorly the blood-brain barrier in dogs (Bonn, 1972; Granville, 1974). I already pointed to the fact, that neither propranolol nor practolol have an affinity to the BR. Of course, β-blockers are able to diminish some somatic manifestations of anxiety, like tachycardia and tremor, and therefore have proven useful in many situations.

Fenobam (McN-3377). Animal studies and uncontrolled clinical trials indicated that this urea derivative may possess weak anxiolytic activity without sedation. A recent double blind study fails however to confirm the previous findings and uncovers side-effects, mainly psycho-stimulant in nature, in 40% of the patients (Friedmann, 1980).

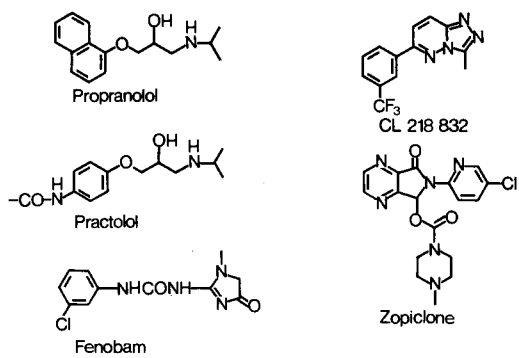


Fig. 11. Other structures.

In addition, the negative 3H-diazepam binding results in vitro make a benzodiazepine-like activity appear very improbable.

Zopiclone (RP-27267) (Jeanmart, 1978). Zopiclone is claimed to be the first new structure possessing the characteristic pharmacological properties of a 1,4-benzodiazepine (Serradell, 1979). Accordingly, the in vitro 3H-diazepam binding results show a moderate affinity to the BR. Zopiclone, which apparently has only mild anxiolytic activity is being developed as a sleep inducer and is stated to be comparable to nitrazepam in humans.

Triazolo-pyridazines (Rodger, 1976). While Zopiclone, as we noticed, shares its pharmacological properties with the 1,4-benzodiazepines, a representative compound of the triazolo-pyridazine series CL-218872 seems to maintain only the anti-conflict and anti-metrazol activities, while having weak sedative and muscle relaxant components (Lippa, 1979). The affinity to the BR in vitro is low if compared with diazepam. Great interest has been aroused by the interpretation of the test results given by the Lederle researche: Adopting the newest notions about a possible differentiation of the BR's (Klepner, 1979), they assume that one of the two or more BR's represents the "anxiolytic" receptor and infer that CL-218872 is a selective anxiolytic interacting specifically with the latter. By contrast, the 1,4-benzodiazepines would not discriminate between the receptors. No clinical work confirming the "pure" anxiolytic properties of CL-218872 has yet appeared.

CONCLUSIONS

The chance discovery of the anxiolytic 1,4-benzodiazepines followed by their widespread therapeutic use, have demonstrated how great the demand for such agents is. But only in recent years, aspects of their possible mechanism of action have begun to be elucidated. The perspectives for drug research in this area are rapidly improving. New biological methods and insights open new opportunities toward a rational approach. Medicinal chemists are obtaining the means to better differentiate the properties of their new compounds and design new anxiolytic structures. As a consequence the development of new and more selective drugs can reasonably be expected in a near future.

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Trans-N-Alkyl-6, 7-Dihydroxyoctahydrobenzo[g]-Quinolines: Apomorphine Congeners Lacking the Non-Oxygenated Aromatic Ring

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ABSTRACT

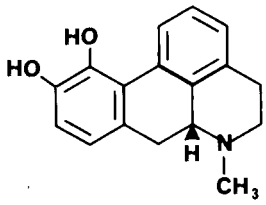
A synthetic sequence leading to the title compounds has been devised. The compounds are related to the dopaminergically active 2-aminotetralins, as well as to apomorphine, and they present the dopamine moiety pharmacophore in the so-called α-conformation. Nitrogen substituents are those which have been demonstrated previously to confer interesting/significant biological effects. High central dopaminergic potency and activity were demonstrated in several animal models. Structure-activity aspects are discussed.

KEYWORDS

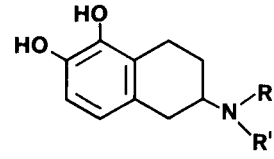
Central dopaminergic effects; α-conformer of dopamine; apomorphine congeners; rigid dopamine congeners.

INTRODUCTION

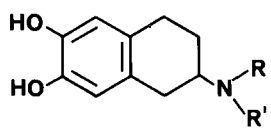
Past work in this laboratory has addressed the chemistry and pharmacology (especially dopaminergic agonist effects) of apomorphine 1 and certain of its congeners and of fragments of the apomorphine molecule, specifically 5,6-dihydroxy-2-aminotetralins ("A-5,6-DTN", 2). These compounds display a broad spectrum of central and peripheral dopaminergic effects.



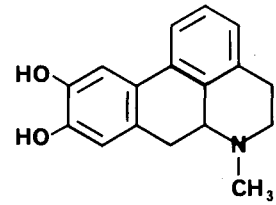
Apomorphine 1



A-5,6-DTN 2

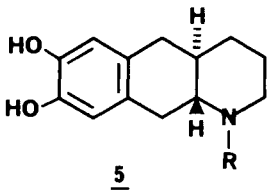


A-6,7-DTN 3



Isoapomorphine 4

A-6,7-DTN derivatives (3), which are hydroxyl group positional isomers of the 5,6-dihydroxytetralins, have been found (McDermed and co-workers, 1975; Cannon and co-workers, 1977) to exhibit high dopaminergic potency and activity in several central and peripheral test models. However, the A-6,7-DTN systems (3) show a different spectrum of dopaminergic effects from the A-5,6-DTN systems (2), and it seems likely that the pharmacologic effects produced by the A-6,7-DTN derivatives result from interactions with different populations of dopamine receptors, both centrally and peripherally, than are involved in the effects produced by apomorphine and A-5,6-DTN derivatives. It has been appealing to attempt to categorize dopamine receptors on the basis of their response or lack of response to the two types of hydroxylation patterns of the agonist molecules (Cannon, 1975). In stereotypy production in rats, it has been suggested (Cannon and co-workers, 1977) that the OH-positional pattern found in apomorphine and in A-5,6-DTN may be preferred for dopamine agonist activity in the caudate-putamen. Further, the hyperactivity and stereotypy effects produced by A-6,7-DTN derivatives may be a reflection of a nonspecific action on presynaptic uptake mechanisms (Horn, 1974), or even an action on the release of neurotransmitter. Goldberg and co-workers (1978a, b) have demonstrated that dopamine causes vasodilation in the canine renal vascular bed, by action on a specific peripheral dopamine receptor. Several compounds that are very potent and active in central nervous system assays are inert in the renal vascular preparation, and only a relatively few synthetic dopamine homologs/congeners have been found which are active in the canine renal vascular bed: some N-alkyl homologs of dopamine itself; the primary amine and some N-alkyl homologs of A-6,7-DTN (3); apomorphine (a weak partial agonist); and N-n-propylnorapomorphine (more potent than apomorphine, and a full agonist) (Goldberg, Volkman, and Kohli, 1978a). Derivatives of A-5,6-DTN are inert in the canine renal vascular assay. Isoapomorphine (4) is enigmatic, in that it is virtually inert in all of the central and peripheral dopaminergic assays in which it has been evaluated (Neumeyer and co-workers, 1973; Cannon and co-workers, 1977), including the canine renal vascular assay (Goldberg and co-workers, 1978b), even though it bears the same chemical relationship to A-6,7-DTN that apomorphine bears to A-5,6-DTN. There is a seeming inconsistency with respect to preference of dopamine receptors for the pattern of agonist benzene ring hydroxylation. Goldberg and co-workers (1978b) have proposed that the topography of the canine renal vascular dopamine receptor is such that apomorphine, A-6,7-DTN, and dopamine can bind appropriately to trigger a response, but A-5,6-DTN and isoapomorphine cannot bind optimally. It was suggested that the unsubstituted benzene ring of isoapomorphine sterically inhibits proper alignment of the molecule with the receptor. Thus, the dramatic difference in dopaminergic renal vascular activity between isoapomorphine (inert) and A-6,7-DTN (extremely potent and active) could be rationalized. A series of N-alkylated-(±)-trans-7,8-dihydroxy-octahydrobenzo[g]quinolines (5) has been prepared (Cannon and co-workers, 1980a), which represents a fragment of the isoapomorphine molecule lacking the unsubstituted aromatic ring, and which is a structural link between isoapomorphine and the A-6,7-DTN systems.



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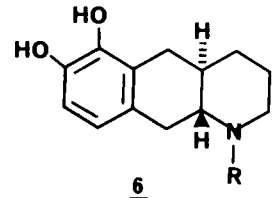


6

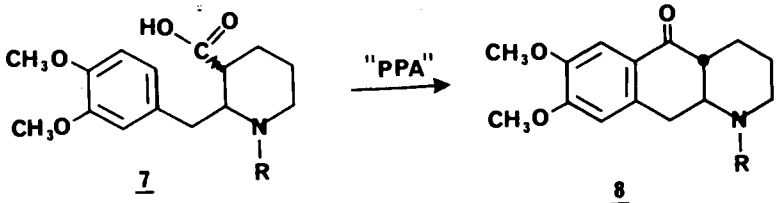
R=H; CH_3; C_2H_5; n-C_3H_7

Like isoapomorphine, the compounds 5 are inert in central nervous system dopaminergic assays in rodents (stereotypic behavior; rotational behavior; hyperactivity; climbing behavior); they are likewise inert in peripheral dopaminergic assays (inhibition of the cardioaccelerator nerve in the cat; dilation of the canine renal vasculature).

It seemed essential to complete the series of apomorphine-aminotetralin-linear benzoquinoline derivatives by preparation of the (±)-trans-6,7-dihydroxy-octahydrobenzo[g]-quinoline ring system 6, bearing oxygen substituents in the pattern found in apomorphine and in A-5,6-DTN.

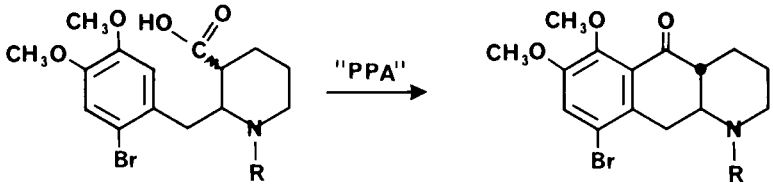
CHEMISTRY

A first approach to the synthetic goal was the well-established literature method (Walsh and Smissman, 1974), utilized for the isomeric 7,8-dioxygenated systems (7 → 8):



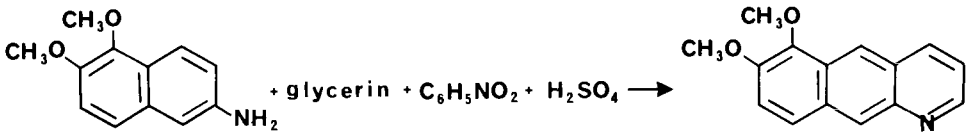
7 → 8

Walsh and Smissman (1974) reported that in this cyclization with polyphosphoric acid, the pathway of the cyclization was such that only the trans-fused product was obtained. Application of this route to the target system 6 presented complications, in that it seemed necessary to introduce a blocking moiety into position 6 of the benzene ring (structure 9), to direct cyclization into the sterically less favored "ortho" position (structure 10) and to prevent cyclization into the alternate and sterically more favored "ortho" position, which would give rise to a product having the wrong pattern of hydroxylation. This cyclization provided extremely poor yields (less than 5%) of tricyclic material, which was not homogeneous; the polyphosphoric acid appeared to effect partial cleavage of the methyl ether groups (Goldman, 1977), and this synthetic approach was abandoned.



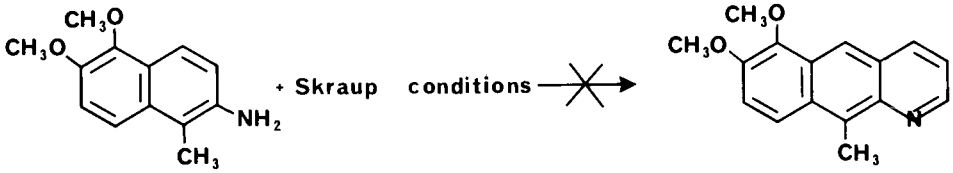
9 10

Skraup cyclization procedures using 5,6-dimethoxy-2-naphthylamine (11) were considered:



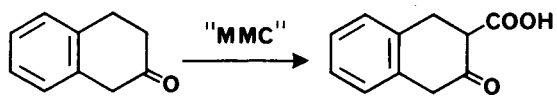
11 12

It seemed possible to effect reduction of the linear benzoquinoline 12 to afford cis- and trans-octahydro systems (6). However, it is well documented (Manske and Kulka, 1953) that 2-naphthylamines undergo Skraup cyclization into the 1-position, to afford as the exclusive tricyclic product the angularly annelated benzo[f]quinoline. Appropriate blocking of position 1 of 2-naphthylamine directs Skraup cyclization into the linear system (Huisgen, 1948), but in the present work, treatment of 1-methyl-5,6-dimethoxy-2-naphthylamine 13 under a variety of Skraup cyclization conditions led invariably to intractable tars.



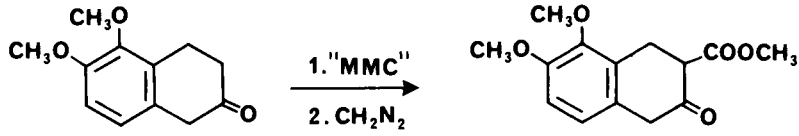
13 14

Pelletier and co-workers (1966) found that treatment of some 2-tetralones with magnesium methoxy carbonate ("MMC") gave 2-tetralone-3-carboxylic acids 16 exclusively, albeit in poor yields (12-26%):



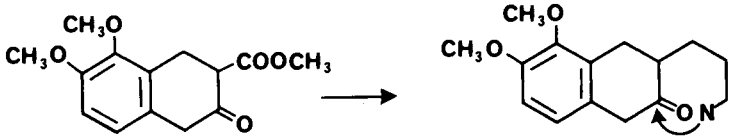
15 16

In the present work, this reaction has been applied (inter alia) to 5,6-dimethoxy-2-tetralone (17), and by optimization of reaction and workup conditions, 50-60% yields of the methyl ester 18 have been achieved (Beres and Cannon, 1979).



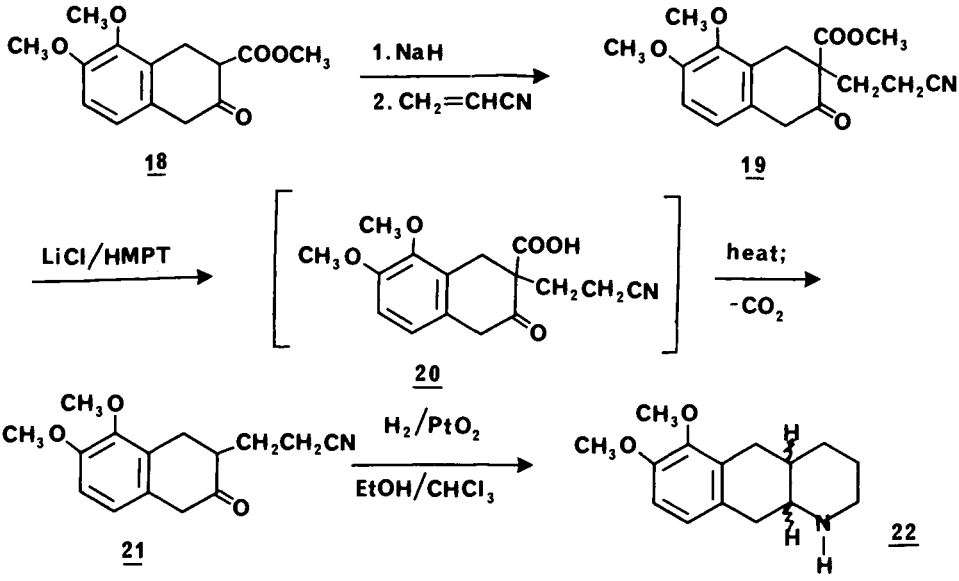
17 18

This reaction is of preparative value, and it was proposed to utilize 3-carbomethoxy-5,6-dimethoxy-2-tetralone (18) as the starting material for formation of a linear octahydrobenzoquinoline. The starting material 18 bears functionalities at carbons 2 and 3, which should be useful in the creation of the necessary piperidine ring, as illustrated in the following hypothetical steps:



18

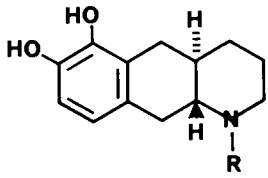
Accordingly, the following sequence has been achieved:



18 19 20 21 22

The overall yield from 18 to the tricyclic product 22 was approximately 10%. Infrared and nuclear magnetic resonance spectral analysis were utilized to establish that the stereochemistry of the material that was isolated from the catalytic reduction step (21 → 22) is the trans-fused isomer, as shown in structure 6. All attempts to isolate cis-fused product failed. However, it cannot be stated that only the trans-ring fusion occurred in the conversion of 21 to 22.

The nitrogen of 22 was alkylated with groups which experience has taught provide interesting/significant pharmacologic effects. Finally, the ether links were cleaved with 48% HBr, to provide a series of racemic compounds (6a-d) for pharmacological testing.



· HBr

6a R = H

6b R = CH_3

6c R = C_2H_5

6d R = n-C_3H_7

Details of the synthetic aspects of this work will be published elsewhere (Cannon and coworkers, 1980b).

PHARMACOLOGY

Emesis in Dogs

The compounds 6b-d are potent emetics in dogs, as shown in TABLE 1.

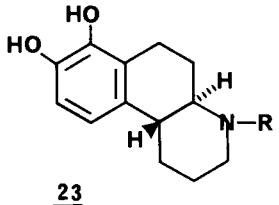
TABLE 1 Emesis in Dogs

Compd. no.	ED_{50}, μmol/kg, s.c.
6b	0.03
6c	0.003
6d	0.003
R-(-)-apomorphine	0.142
(±)-5,6-dihydroxy-2-di-n-propylaminotetralin	0.002[a]

[a] Minimum effective dose, from McDermed, McKenzie, and Phillips (1975).

Inhibition of DOPA Accumulation in Rat Striatal Tissue

Walters and Roth (1976) have suggested that inhibition of striatal DOPA accumulation following administration of apomorphine reflects stimulation of presynaptic dopamine autoreceptors, and Baring and co-workers (1978) have shown that this effect does not involve feedback via the nigrostriatal pathway. In contrast to dopamine receptors that mediate rotational behavior, striatal dopamine autoreceptors are not believed to be associated with adenylate cyclase (Kebabian and Calne, 1979). It has been reported (Rusterholz and co-workers, 1980) that A-5,6-DTN (2) and its N-alkyl homologs and the octahydrobenzo[f]-quinolines (23), which have the apomorphine hydroxylation pattern, are equipotent to (and in some instances, are more potent than) apomorphine in inhibition of striatal DOPA accumulation, as contrasted with A-6,7-DTN derivatives (3), the linear octahydrobenzoquinoline series (5), and the angular congener 30, which were decidedly less active than apomorphine and, in the case of 5, inert.



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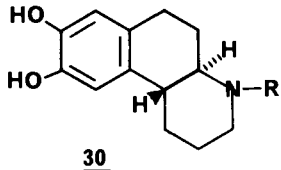


30

In the present study, the tertiary amines 6b-d at a dose level of 1 mg/kg were somewhat more active than an equivalent dose of apomorphine in inhibition of striatal DOPA accumulation.

Stereotypy in Mice

Sniffing, gnawing, biting, and licking in mice was produced by subcutaneous dosage of 1 mg/kg with apomorphine and with the N-ethyl- (6c) and n-propyl (6d) derivatives. At 10 mg/kg, the N-methyl homolog (6b) also produced stereotypy.

Rotational Behavior in Rats

Rats were unilaterally lesioned in the substantia nigra with 6-hydroxydopamine. Apomorphine and the N-methyl- (6b) and n-propyl (6d) linear benzoquinoline derivatives, administered subcutaneously, produced contralateral circling, indicative of a direct central dopaminergic effect (Ungerstedt, 1971). The n-propyl homolog 6d was somewhat more potent than apomorphine, and the N-methyl 6b was less potent than apomorphine. However, the N-ethyl derivative 6c elicited no rotational response at doses approximately ten times those required for good response to apomorphine and to the n-propyl derivative 6d. The rotational inactivity of the N-ethyl homolog 6c is difficult to rationalize on chemical structural grounds. Perhaps a proposal of Costall, Naylor, and co-workers (1977a, b) can be invoked: that gross, centrally mediated rotational effects produced by dopaminergic drugs may result from more than one physiological mechanism, involving more than one population of dopamine receptors, having different agonist structural requirements.

Hypothermia in Mice

At dose levels of 1 mg/kg, subcutaneously, all four of the test compounds (6a-d) produced hypothermia in mice. The potency in this assay increased with the size of the N-alkyl chain. The n-propyl homolog (6d) produced a greater temperature drop of much longer duration than apomorphine at the same dose level.

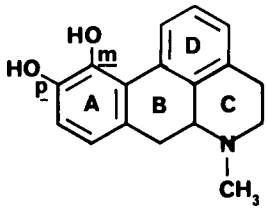
Renal Blood Flow in Dogs

In a peripheral assay for renal blood flow increase, only one compound, the n-propyl derivative (6d), manifested a marked dopamine-like effect, having a potency ratio to dopamine of approximately 0.2 . This value represents a tenfold increase in potency over what has been reported for N-n-propylnorapomorphine (Goldberg, Volkman, and Kohli, 1978a). It has been previously noted that the isomeric linear octahydrobenzoquinoline (5: R = n-C_3H_7) was inert in this assay.

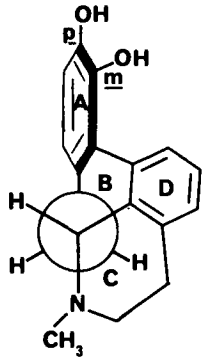
STRUCTURE-ACTIVITY CONSIDERATIONS

The secondary amine 6a exhibited low potency and activity in most of the assays, which is consistent with the finding (Koch and co-workers, 1968) that the secondary amine norapomorphine has only weak central dopamine-like effects. The subject compounds 6b-d produce effects much like apomorphine and its N-alkyl homologs in all of the assays performed, but the linear benzoquinolines tend to be more potent than the corresponding apomorphine derivatives, whose effects have been described earlier (Koch and co-workers, 1968). It is appealing to speculate that the "extra" benzene ring of the apomorphine system exerts a negative effect on agonist-receptor interactions. As has been noted (Cannon, 1975), the N-n-propyl substituent seems to confer unique biological potency upon dopaminergic agonist molecules. It is concluded that dopamine receptors exhibit a positive discrimination toward N-n-propyl groups, and that optimal receptor-agonist interaction occurs when the nitrogen of a dopaminergic agonist bears at least one n-propyl group.

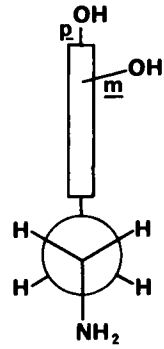
Molecular models indicate that the conformation of the dopamine moiety within the R-(-)-apomorphine molecule (1) is as indicated in the Newman projection 24.



Apomorphine 1



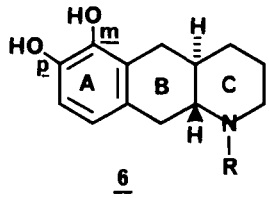
24



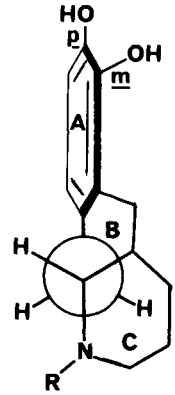
25

The apomorphine molecule presents the dopamine moiety with the catechol ring and the amino group in an antiperiplanar disposition, with the plane of the catechol ring approaching coplanarity with the ethylamine side chain. (The catechol ring appears skewed 25-30° counterclockwise out of perfect coplanarity with the ethylamine side chain.) . However, this steric disposition seems to be within the permissible steric range for the α-conformer (25) of dopamine, which has been proposed (Cannon, 1975) to be of significance in reactions of dopamine with certain of its receptors. In the α-conformer, the catechol ring is depicted as being coplanar with the ethylamine side chain, and the "meta"-OH is on the edge of the ring nearer to the side chain.

A molecular model of the (±)-trans-octahydrobenzo[g]quinoline system 6 reveals that the overall molecule, lacking the aromatic D ring of apomorphine, is not as sterically restrained as apomorphine, but nevertheless it has a significant degree of molecular rigidity imposed upon it by the trans- geometry of ring fusion. The most stable steric disposition of trans- 6 seems to be one in which the B ring exists as a somewhat distorted half chair, and the C ring is a chair. Other "flip" conformations for the C ring involve primarily a change from a chair to a boat, and molecular models suggest that this type of flip has little effect upon the spatial disposition of the dopamine moiety within the molecule. A Newman projection (26) of the presumed most stable conformer of trans-octahydrobenzo[g]quinoline (6) illustrates that the dopamine moiety is held in an almost perfect α-conformation, with the plane of the catechol ring only 15-20° out of coplanarity with the ethylamine side chain.



6

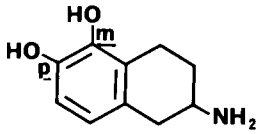


26

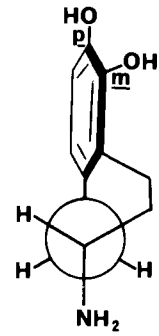
Structures 6 and 26 are drawn to represent the same absolute configuration of the chiral center as is found in the analogous carbon in R-(-)-apomorphine. Saari, King, and Lotti (1973) showed that dopaminergic activity of apomorphine resides exclusively in the enantiomer shown (structure 1) and it is assumed that only one of the enantiomers in (±)-6 (presumably having the absolute configuration shown) is active. Thus, the biological potencies reported for the derivatives of (±)-6 are half of that expected for the pure active enantiomer of each compound.

It has been reported (Cannon, 1975, 1979) that the biologically significant conformer of dopaminergic 2-aminotetralins, typified by A-5,6-DTN (2), is that one in which the amino group is attached to the ring by a pseudoequatorial bond. In this conformer, the plane of

the catechol ring is approximately 15-20° out of coplanarity with the ethylamine side chain of the dopamine moiety, illustrated in Newman projection 27.



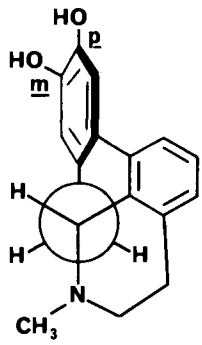
A-5,6-DTN 2



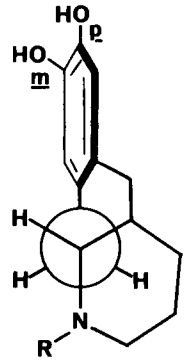
27

Hence, the 2-aminotetralins are structurally very similar to the octahydrobenzo[g]-quinolines, in their close approach to the α-conformational extreme of dopamine. This is reflected in the close similarity of biological potency and activity of A-5,6-DTN homologs and the octahydrobenzo[g]quinolines (6). Insofar as gross visual measurements of torsion angles in models of these molecules are valid and accurate, it may be inferred from them that the biological activities and potencies of the trans-6,7-dihydroxy-octahydrobenzo[g]-quinolines 6a-d relative to those of apomorphine may reflect a better degree of molecular complementarity to the dopaminergic receptor(s) by the compounds 6.

Isoapomorphine (4) and the trans-7,8-dihydroxy-octahydrobenzo[g]quinoline system 5 differ from their hydroxyl group positional isomer counterparts 1 and 6 only in the position of the "meta"-OH group, which is illustrated in the Newman projections 27 and 28.

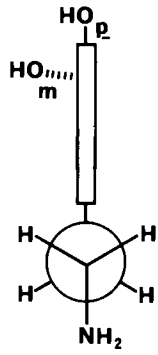


Isoapomorphine



trans-7,8-dihydroxy-octahydrobenzo(g) quinoline

28



Dopamine: beta conformer

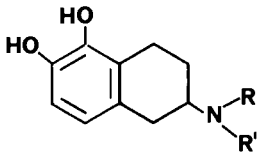
29

trans-N-Alkyl-6,7-dihydroxyoctahydrobenzo[g]-quinolines

These are frozen congeners of the β-conformer of dopamine, in which the benzene ring is coplanar with the ethylamine side chain, and the "meta"-OH is on the edge of the ring away from the side chain.

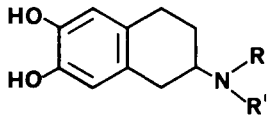
The following structures summarize the ring systems and hydroxylation patterns to which our research efforts have been addressed:

ALPHA CONFORMERS:

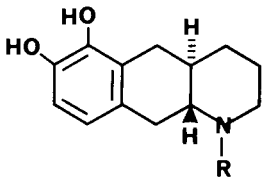


A-5,6-DTN 2

BETA CONFORMERS:



A-6,7-DTN 3



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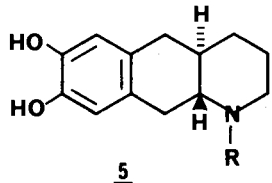

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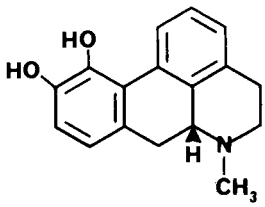
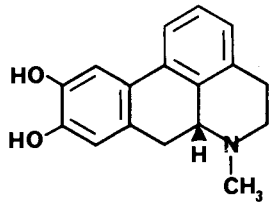
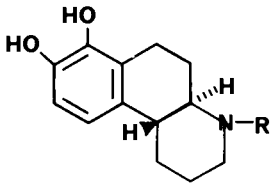


Apomorphine 1



Isoapomorphine 4



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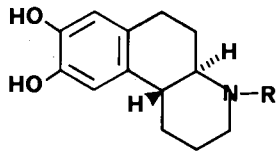

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In comparing the dopaminergic actions (both central and peripheral) of the various series of rigid and semi-rigid congeners of the two conformational extremes of dopamine, it is striking that, in general, members of all of the α-conformer systems exhibit a high degree of activity and a similar spectrum of central and peripheral dopaminergic agonist effects, and members of all ring systems are often of high potency. Pharmacologically, these four ring systems are quite similar. In contrast, of the four β-conformer systems, only A-6,7-DTN homologs uniformly display a spectrum of central and peripheral dopaminergic actions. The linear benzoquinoline 5 and isoapomorphine (4) homologs are virtually inert in all of the assays (both central and peripheral) in which A-6,7-DTN is active and potent. Compound 30 is intriguing, in that it possesses some specific, highly potent peripheral dopaminergic actions (Cannon and co-workers, 1980a) and it has moderate ability to inhibit striatal DOPA accumulation (Rusterholz and co-workers, 1980), but it is inert in all other central nervous system dopamine assays in which it was tested (stereotypy induction, hyperactivity, climbing behavior in rodents). It must be concluded that, whereas biological effects for rigid and semi-rigid congeners of the α-conformer of dopamine are predictable and consistent, structure-activity relationships for β-conformers are more subtle and much less predictable.

ACKNOWLEDGEMENT

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Dopamine Receptor Blockade by Substitute Benzamide Drugs

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ABSTRACT

The pharmacological and therapeutic effects of the substituted benzamide group of drugs are thought to be related to blockade of central and peripheral dopamine receptors. Nevertheless individual members of the group exhibit properties which are not entirely consistent with such a concept and potency ratios between the different substituted benzamides vary considerably from one test model to another. These anomalies are discussed against a background of contemporary knowledge of the distribution and function of mammalian dopaminergic systems in an attempt to demonstrate the importance of such factors as the blood-brain barrier and the existence of both pre- and postsynaptic dopamine receptors in the interpretation of the biochemical and pharmacological properties of this group of drugs. The problems presented to the pharmacologist by these and other factors in his attempts to predict the therapeutic potential of new compounds and to the medicinal chemist in his attempts to correlate structure with activity are also considered. A variety of commercialized substituted benzamides ranging chronologically from metoclopramide to clebopride are used as examples together with several new entities which are as yet still under investigation.

KEYWORDS

Substituted benzamides; o-anisamides; orthopramides; dopaminergic mechanisms; dopamine receptor antagonists; pre-and postsynaptic receptors.

INTRODUCTION

During the last two decades, dopamine, the immediate precursor of the sympathetic and central neurotransmitter, noradrenaline, has become recognised, albeit sometimes putatively, as a fully fledged neurotransmitter in its own right, acting on both sides of the blood-brain barrier. One group of compounds that has contributed much to recent interest and research in dopaminergic mechanisms is that of the substituted benzamides (o-anisamides or orthopramides) especially since they have often produced anomalous and atypical pharmacological and clinical

effects for compounds which supposedly block dopamine receptors. Thus, although of the first two such compounds introduced into clinical practice only metoclopramide shows significant activity in the classical behavioural animal models used for detecting antidopaminergic neuroleptics (Elliott and co-workers, 1977; Puech and others, 1978), it is only its stablemate, sulpiride, that shows clinically useful antipsychotic activity (Nakra, Bond and Lader, 1975; Mielke and colleagues, 1977). Nevertheless, the use of metoclopramide as an antiemetic and inducer of gastrointestinal motility has been associated with some neuroleptic-like extrapyramidal dystonic reactions (Robinson, 1973) and, as an additional complication, sulpiride has also been described as having antidepressant properties (Niskanen and co-workers, 1975), a claim approaching, at least superficially, pharmacological heresy for an antidopaminergic neuroleptic.

Faced with such confusion, medicinal chemists and pharmacologist engaged in the search for new therapeutically useful drugs might well be excused for a certain lack of enthusiasm for working with substituted benzamides, and this may well have contributed to the evident delay between the appearance of metoclopramide and sulpiride and their successors. Nevertheless, although nobody appears to dominate the situation sufficiently to attempt, at least publicly, to correlate chemical structure with biological activity, the last few years have seen an almost explosive increase in the number of substitute benzamides appearing in the literature and in the WHO lists of approved names. Apart from the variations in the substituents on the benzamide nucleus, these compounds can be divided into five main groups where the amide side chains are respectively alkylaminoalkyl, 2-pyrrolidinylmethyl, 3-pyrrolidyl, 4-piperidyl and piperazinyl (Table 1).

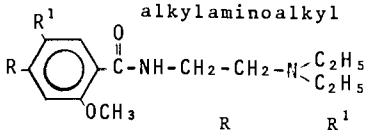
Since this sudden appearance of new generations of substituted benzamides probably reflects the recent advances made in dopamine receptor research, it would seen profitable to reconsider the anomalies existing between the pharmacological activity and therapeutic use of some of these compounds against a background of contemporary knowledge of the distribution and function of mammalian dopaminergic systems, in an attempt to rationalize the extrapolation of experimental data to man.

DOPAMINERGIC MECHANISMS

Although a new nomenclature describing seven different central dopaminergic projections has been recently published (Lindvall and Björklund, 1978), according to the more classical description most of the dopaminergic axons ascend via three distinct pathways. The nigrostriatal tract fibres originate mainly from cell bodies in the substantia nigra, ascend through the lateral and mid-hypothalamus and then fan out to innervate the neostriatum. The mesolimbic tract fibres originate principally from cell bodies surrounding the interpeduncular nucleus, ascend to innervate the nucleus accumbens and olfactory tubercle of the limbic forebrain and continue into the limbic cortex, the hippocampal formation and the frontal cortex. The tuberoinfundibular tract fibres originate in the arcuate nucleus of the hypothalamus and ascend into the median eminence where the terminals are in close relation to the portal vessels and other types of nerve endings, some of which contain the anterior pituitary releasing factors. The possible physiological functions of these and other central and peripheral systems potentially mediated by dopaminergic mechanisms are indicated in Table 2.

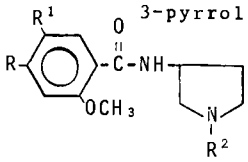
TABLE 1 Named or Described Substituted Benzamides Subdivided according to the Amide Side Chain

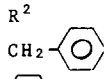
alkylaminoalkyl



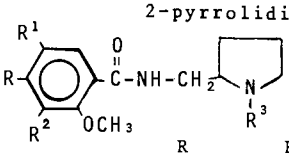
	R	R^1
Metoclopramide	NH_2	Cl
Bromopride	NH_2	Br
Tiapride	H	SO_2CH_3

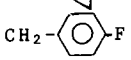
3-pyrrolidinyl



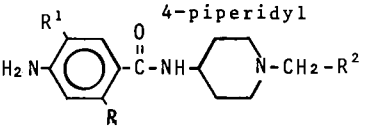
	R	R^1	R^2
YM-08050	CH_3NH	Cl	
AHR 5859	NH_2	Cl	
AHR 6092	H	SO_2NH_2	

2-pyrrolidinylmethyl



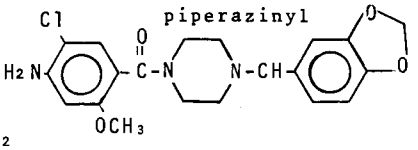
	R	R^1	R^2	R^3
Sulpiride	H	SO_2NH_2	H	C_2H_5
Sultopride	H	$SO_2C_2H_5$	H	C_2H_5
Sulmepride	H	SO_2NH_2	H	CH_3
Prosulpride	H	SO_2NH_2	H	C_3H_7
Veralipride	H	SO_2NH_2	OCH_3	$CH_2CH{=}CH_2$
Alizapride	-NH - N = N-		H	$CH_2CH{=}CH_2$
Cipropride	H	SO_2NH_2	H	
Flubepride	H	SO_2NH_2	H	

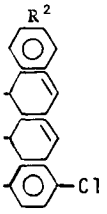
4-piperidyl



	R	R^1
Clebopride	OCH_3	Cl
Alepride	$OCH_2CH{=}CH_2$	Cl
Cinitapride	OC_2H_5	NO_2
Broclepride	OCH_3	Br

piperazinyl





Peralopride

In the central nervous system dopaminergic stimulation results in increased motor activity and stereotyped behaviour in animals and psychotic states in man (Barnett, 1975; Wallach, 1974) and although there is some controversy over the relative importance of the nigrostriatal and mesolimbic systems in the mediation of these effects it is generally accepted that the therapeutic activity of the major tranquillizers is associated with a blockade of dopamine receptors in the latter whereas the catalepsy and dyskinetic reactions seen with these drugs results from a blockade of dopamine receptors in the former.

In the tuberoinfundibular tract, dopamine either is, or causes the release of, the prolactin inhibitory factor in the anterior pituitary gland cells (McLeod and Lehmeyer, 1974) so that dopaminergic stimulants result in decreased secretion of prolactin and antidopaminergic drugs in increased secretion, with consequent influences on oestrus cycles and mammary gland development. Of great importance for the interpretation and extrapolation of pharmacological results is the fact that the axons of the tuberoinfundibular system extend out into an environment not protected by the blood-brain barrier (Baumgarten and colleagues, 1972).

TABLE 2 Mammalian Dopaminergic Mechanisms

Site	Potential function in control of:
Central Nervous System	
Nigrostriatal tract	Voluntary movement
Mesolimbic tract	Emotional response to stress Motor activity? Body temperature?
Tuberoinfundibular tract	Prolactin secretion, Gonadotropins?
Hypothalamus	Appetite, Body temperature?
Chemosensitive trigger zone of the 4th ventricle	Nausea and vomiting Blood-pressure?
Retina	Response to light
Olfactory tubercle	Response to odour
Peripheral Sites	
Sympathetic ganglia	Ganglionic transmission
Carotid bodies	Reflex responses of blood pressure and respiration
Blood vessels (superior mesenteric, renal and coeliac beds)	Tone
Oesophagus	Tone and motility
Lower oesophageal sphincter	" " "
Stomach	" " " , acid secretion
Duodenum	" " "
Colon	" " "
Pancreas	Alkaline secretion

Central dopaminergic stimulation has also been associated with suppression of feeding (Heffner and co-workers, 1977) and in this case the site of action appears to be the incertohypothalamic dopaminergic pathway (Leibowitz, 1978). An hypothalamic site of action is also probable for the hypothermia resulting from the systemic or central administration of dopaminergic stimulants in most species studied (Barnett, 1975; Cox and Lee, 1977) although it has been suggested that the receptors involved are in the mesolimbic system (Yeyuda and Wurtman, 1975). Centrally mediated dopaminergic hypotension may also be located in the hypothalamus or midbrain (Barnett and Fiore, 1971), although it may also be related, at least in part to stimulation of the dopamine receptors in the area postrema of the 4th ventricle (Bolme and co-workers, 1977).

In those species capable of vomiting such as cat, dog, pigeon and man, dopaminergic stimulation of the chemosensitive trigger zone of 4th ventricle (area postrema) induces emesis (Barnett, 1975). As with the pituitary it seems probable that the chemosensitive trigger zone is outside of the blood-brain barrier since whereas apomorphine induces both vomiting and stereotyped convulsive pecking in the pigeon, the quaternized apomorphine methiodide, which cannot penetrate the blood-brain barrier, only induces vomiting (Cheng and Long, 1974).

Functionally, histochemically and embryologically the retina is part of the central nervous system and contains dopamine as the predominant amine located in the amacrine cells at the junction of the inner nuclear and inner plexiform layers, which make multiple synaptic connections with a variety of cell types (Ehringer, 1977). Although the physiological rôle of these dopaminergic synapses is unclear both synthesis and release of the dopamine is increased on exposure to light (DaPrada, 1977).

Dopamine may also be involved in the sensory response to odour since there are dopamine containing periglomerular cells in the olfactory tubercle (Halász and co-workers, 1977) and,as described above, this brain region is innervated by the mesolimbic dopaminergic projection.

Outside of the central nervous system dopaminergic interneuronal mechanisms inhibit transmission in sympathetic ganglia by reducing the presynaptic release of acetylcholine and/or its postsynaptic action (Libet, 1977; Koslow, 1977) and inhibit the sensitivity of the chemoreceptive nerve endings of the glomus cells of the carotid body (McDonald, 1977). Both of these mechanisms can obviously influence blood pressure and, especially in the case of the carotid bodies, respiration, but blood vessels, particularly those of the superior mesenteric, renal and coeliac vascular beds, also contain dopamine receptors, stimulation of which induces vasodilatation and a consequent hypotension (Goldberg, 1972).

Dopamine also causes relaxation of the smooth muscle at virtually all levels of the gastrointestinal tract although for the moment specific receptors have only been proposed in the oesophagus, the lower oesophageal sphincter and the stomach, and dopaminergic mechanisms have been implicated in the physiological regulation of the contractions of the oesophagus and relaxation of the stomach during swallowing and distension (De Carle and Christensen, 1976; Mukhopadhyay and Weisbrodt, 1977; Valenzuela, 1976); it has also been suggested that dopaminergic interneurones might modify 5-hydroxytryptaminergic transmission in the myenteric plexi in much the same way as cholinergic transmission is modified in the sympathetic ganglia (Schulz and Goldstein, 1973; Roberts, 1979). Finally, dopamine may also play a rôle in the control of digestive se-

cretions since it decreases gastric acid secretion (Tittobello and co-workers, 1978) and increases pancreatic alkaline secretion (Hashimoto and co-workers, 1971).

Needless to say, in all of the systems discussed, the effects of dopamine are mimicked by other dopamine receptor agonists such as apomorphine and piribedil, and are specifically inhibited by dopamine receptor blocking drugs such as haloperidol, pimozide and, although not consistently, the substituted benzamides.

The actual mechanisms involved in dopaminergic transmission are illustrated in Fig. 1. Dopamine is synthesized from L-tyrosine via L-dopa, a process which can be inhibited by α-methyltyrosine (inhibiting tyrosine hydroxylase) or by benserazide or carbidopa (inhibiting L-dopa decarboxylase). Newly synthesized dopamine is taken up into a labile or functional store and if not released is transferred to a bound or reserve store (Glowinski, 1975). Presynaptic impulses cause the release of

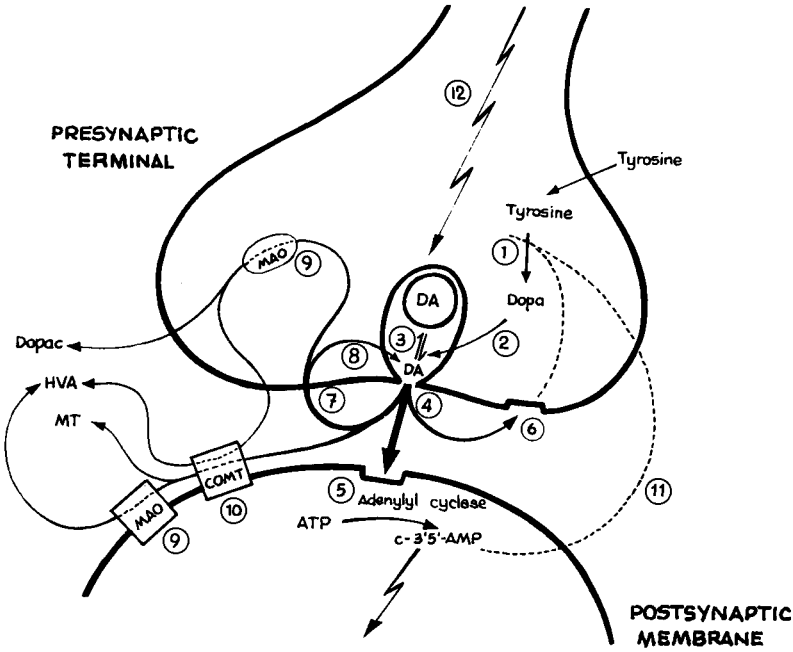


Fig. 1. Schematic representation of a dopaminergic neurone

MAO, monoaminoxidase; COMT, catechol-O-methyltransferase; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MT, methoxytyramine, 1) and 2) enzymatic synthesis from tyrosine to dopamine; 3) accumulation in labile and then bound store; 4) release; 5) interaction with postsynaptic receptors; 6) interaction with presynaptic receptors; 7) reuptake into neurone; 8) reuptake into store; 9) metabolism by MAO; 10) metabolism by COMT; 11) intraneuronal feedback; 12) presynaptic stimulus. For further explanation, see text.

dopamine into the synaptic cleft and these impulses are blocked by γ-hydroxybutyrate or its precursor, γ-butyrolactone. The liberated dopamine stimulates postsynaptic receptors (one of which appears to be an adenylyl cyclase sensitive to dopamine, Kebabian, 1978), resulting in the initiation of the biological responses classified as dopaminergic already described, and also in the control of synthesis and liberation of more dopamine by a process of intraneuronal feedback (Carlsson and Lindquist, 1963), inhibited by kainic acid which destroys the post synaptic perikaryon (Coyle and Schwartz, 1976; McGeer and McGeer, 1976), or by γ-butyrolactone which inhibits transmission in the presynaptic neurone. The process of synthesis and liberation of dopamine is also inhibited by stimulation of the presynaptic or autoreceptors (Carlsson, 1975). Much of the evidence supporting the existence of such receptors comes from experiments with apomorphine which in vitro inhibits the synthesis and release of dopamine from striatal slices and synaptosome preparations (Christiansen and Squires, 1974) and in vivo inhibits the synthesis of dopamine at doses well below those required to induce stereotyped behaviour or increased motor activity (Strombon, 1976), even after postsynaptic receptor destruction with kainic acid (Di Chiaro and co-workers, 1977). By a process of reuptake the released dopamine is also returned first of all to the presynaptic neurone (inhibited by amphetamine, benzotropine or nomiphensine) and then to the store (inhibited by reserpine and tetrabenazine). Non stored intraneuronal dopamine is metabolized by monoamine oxidase in the mitochondria to dihydroxyphenylacetic acid (DOPAC) which, on leaving the neurone, is further converted to homovanillic acid (HVA) by catechol-O-methyltransferase. This enzyme also metabolizes released dopamine to methoxytyramine (MT) which in its turn can be converted to HVA by postsynaptic monoamine oxidase. Changes in tissue concentrations of DOPAC and HVA have been taken to indicate the turnover of dopamine, in that the concentration of metabolites reflects the amount of dopamine released, and since dopamine release is controlled by dopamine receptor stimulation, changes in levels of HVA have been used to measure the degree of receptor blockade induced by antidopaminergic drugs. Unless carried out in preparations treated with kainic acid or γ-butyrolactone, however, such measurements will not differentiate between postsynaptic block and presynaptic block.

SUBSTITUTED BENZAMIDES AS DOPAMINE RECEPTOR BLOCKING AGENTS

One of the most basic concepts of dopamine receptor blockade is obviously that the blocking agent should antagonize the actions of dopamine receptor stimulants and Table 3 summarizes the available information on twelve substituted benzamide compounds comparing their potency in a variety of test models using apomorphine as the dopamine receptor stimulant. Any one of these tests may be used as a screen for dopamine receptor blocking activity and it is very evident that any attempt to define structure-activity relationships must also define the test model chosen, since the activities of the different compounds vary from one model to another. Nevertheless it seems clear that this variability is more a consequence of changes in the substituents in the benzamide nucleus than of differences in the side chains. Thus although sulpiride and its analogues are characterized by their loss of activity in the rodent behaviour models, whilst maintaining activity against apomorphine-induced emesis in the dog, this is related more to the 2-methoxy, 5-sulphonamido nucleus than to the 2-pyrrolidinylmethyl side chain in that the compound with the metoclopramide nucleus (2-methoxy, 4-amino, 5-chloro) and the sulpiride side chain is more active than metoclopramide against apomorphine-induced

stereotyped behaviour in the rat (unpublished results, Laboratorios Almirall). The same situations if found in the 3-pyrrolidinyl series where AHR 6092 (with the sulpiride nucleus) is much less active than YM-08050 (with the metoclopramide nucleus) against apomorphine-induced stereotyped behaviour in the rat, and in the generally potent 4-piperidyl series where the introduction of a 2-methoxy, 5-sulphonamido nucleus results in a significant loss of activity in the rodent stereotyped activity models (unpublished results, Laboratorios Almirall).

Since the only dopamine receptor stimulant used in all of the test models cited was apomorphine, there are several possible explanations of the apparent anomalies in antidopaminergic activity.

One hypothesis might be that apomorphine induces some effects that are dopaminergic and others that are not. Certainly in the snail there are dopamine receptors which are not stimulated by apomorphine (Woodruff, 1971), but at the same time neither are they blocked by dopamine receptor antagonists, and for the moment there is no reason to suppose that apomorphine, at the doses and in the models referred to in Table 3, is

TABLE 3 Comparative Activity of Substituted Benzamide Drugs against Apomorphine-induced Effects in Animal Models

Substituted benzamide	Apomorphine - induced					
	Emesis (Dog)	Stereotyped gnawing (rat)	Circling behaviour (rat)	Climbing behaviour (rat or mouse)	Hypothermia (mouse)	Increased Motor activity (mouse)
Alkylaminoalkyl						
Metoclopramide	++	++	++	++	++	++
Tiapride	+	+	+	+[1]	+	+
2-pyrrolidinylmethyl						
Sulpiride	+++	±	-	+	++[2]	+[3]
Sulmepride		+	-	±	++[2]	+[3]
Prosulpride		+	+	+	++[2]	+[3]
Sultopride	+++	++	++	++[1]	++	++
3-pyrrolidinyl						
AHR 5859	+++					
AHR 6092	++	±				
YR 08050	+++	+++				
4-piperidyl						
Clebopride	+++	+++	+++	++	+++	+++[3]
Cinetopride	+++	++		+++	+	
Alepride	+++	++		++	++	
Broclepride	+++	+++	+++	++	+++	

Results taken from Alphin, Droplemann and Gregory, 1978; Costall and co-workers, 1978a; Puech, Simon and Boissier, 1978; Roberts and co-workers, 1978 and unpublished results.
All activities compared with metoclopramide following peripheral administration. One + represents at least a 5 fold difference in potency.
[1]incomplete antagonism, [2]reversal of antagonistic effect at higer doses, [3]potentiation of apomorphine-induced hyperactivity at lower doses.

acting on systems other than dopaminergic ones. Furthermore, similar anomalies are to be found in experimental models not utilizing exogenous dopamine receptor stimulants (Table 4) in that whereas all substituted benzamides tested increase prolactin secretion from the pituitary causing hypertrophy of the mammary glands and also increase cerebral concentrations of HVA, sulpiride-like compounds, in contrast to other substituted benzamides, appear to have only weak cataleptagenic effects. Nevertheless, as is evident from the results presented, the absolute cataleptagenic potency of any one compound varies with the test method chosen and the relative potency amongst a series of compounds depends upon the time allowed between drug administration and measurement of catalepsy (Table 4). Under optimal conditions the potency differances are minimized and sulpiride induces catalepsy at doses which are relatively low, especially considering that its clinical dose is 5x and 100x greater than that of metoclopramide and clebopride respectively.

TABLE 4 Activity of Clebopride, Metoclopramide and Sulpiride in Tests for Antagonism of the Effects of Endogenous Dopamine

Substituted benzamide	Mammatrophic effect[1]	Whole Brain HVA[2]	Catalepsy[3] Wirth(hr after administration)		Janssen(hr after administration)	
Clebopride	4.09	0.144	3.8(2)	1.74(4)	0.55(2)	0.45(4)
Metoclopramide	12.09	3.16	77.4(2)	19.9(3)	9.3(2)	5.5(4)
Sulpiride	18.56	34.67	230.9(2)	54.8(6)	255.0(2)	13.1(4)

[1])ED_{50} mg kg^{-1} p.os 13 days treatment (see Lotz and Krause, 1978)
[2])ED 1/2 max. mg kg^{-1} sub.cut. 2h after drug administration
[3])ED_{50} mg kg^{-1} sub.cut. 2h after drug administration and at time of maximum effect using methods of Wirth and co-workers, 1958 and Janssen and co-workers, 1965). Berga and colleagues, unpublished results.

Another possible hypothesis is that the localization of the different populations of dopamine receptors is such that some are more readily available to some antagonists than to others. In this context the fact that sulpiride has been shown to only poorly penetrate the blood-brain barrier is of obvious significance, and in contrast to its relatively weak central effects following peripheral administration, when injected centrally it is a potent antagonists of dopaminergic stereotyped behaviour (Table 5) and induces intense catalepsy (Honda and co-workers, 1977; Costall, Fortune and Naylor, 1978). Since, as has already been stated, neither the pituitary gland nor the chemosensitive trigger zone of the 4th ventricle are protected by the blood-brain barrier, the potent activity of peripherally administered sulpiride and similar benzamides in inducing hyperprolactinemia and its sequelae, and in protecting against apomorphine-induced emesis in the dog, is readily explained. More difficult to account for is the increase in cerebral HVA content resulting from sulpiride administration since this also occurs in rats and at doses which are ineffective against apomorphine-induced stereotyped behaviour. Since prolactin administration has been shown to increase rat brain HVA, particularly in the mesolimbic areas (Fuxe and colleagues, 1977), it is tempting to suggest that the increase in cerebral HVA content following sulpiride treatment is secondary to the increase in prolactin secretion, but this appears to be improbable because sulpiride still elevates brain HVA in hypophysectomized animals (Jenner and co-workers, 1978).

TABLE 5 Comparative Activity of Clebopride, Metoclopramide and Sulpiride Administered by Different Routes Against Various Effects produced by Stimulation of Central Dopaminergic Mechanisms

Experimental model	Route of administration	ED_{50} values mg kg^{-1} Clebopride	Metoclopramide	Sulpiride
Apomorphine-induced gnawing in the rat	oral[1,2]	2.4	8.7	> 2000
	i.v.[2]	0.06	1.3	> 50
	i.p.[3]	0.3	5.4	> 64
	i.c.v.[4]	-	-	1
Amphetamine-induced gnawing in the mouse	oral[5,6]	5.2	11.4	> 100
	i.v.[5,6]	0.6	9.2	
Apomorphine-induced climbing in the mouse	oral[6]	1.0	2.2	> 160
Apomorphine-induced turning in the mouse	i.p.[7]	0.6	3.0	7.0
Amphetamine-induced hyperactivity in the mouse	i.p.[8]	0.5	3.0	> 40
	i.c.[8]	0.006	0.08	0.002
Apomorphine-induced emesis in the dog	oral[2]	0.04	0.45	0.14
	i.v.[2]	0.01	0.08	0.015
	s.c.[9]	0.0025	0.03	0.002

i.v., intravenous; i.p., intraperitoneal; i.c.v., intracerebroventricular; s.c., subcutaneous; i.c., intracerebral (n. accumbens).

[1])Prieto and co-workers, 1977; [2])Salazar and co-workers, 1978; [3])Jenner and co-workers, 1978; [4])Honda and co-workers, 1977; [5])Roberts and co-workers, 1978; [6])Beckett and co-workers, 1980; [7])Elliott and co-workers, 1977; [8])Costall and co-workers, 1978; [9])Costall and co-workers, 1978a.

Nevertheless, even within the blood-brain barrier the different brain regions involved in the various dopamine receptor models are not equally accessible to all substituted benzamides to the same extent, and there is evidence that striatal and mesolimbic areas are affected differentially (Bartholini, 1976). Whereas, for example, sulpiride, sultopride and clebopride antagonize the hyperactivity induced by dopamine injected directly into both the nucleus accumbens or the caudate-putamen of the rat, metoclopramide and tiapride are only effective against caudate-putamen administered dopamine (Costall and co-workers, 1978a), a finding which offers a possible explanation of the extrapyramidal side effects found in man with metoclopramide in the absence of antipsychotic activity, and which may be related to the usefulness of tiapride against tardive dyskynesias. Similarly, since stereotyped behaviour and climbing behaviour appear to be related to stimulation of striatal dopamine receptors (Ernst, 1968; Protais and colleagues, 1976), increased motor activity to stimulation of mesolimbic dopamine receptors (Pijnenburg and co-workers, 1973), and hypothermia to stimulation of hypothalamic dopamine receptors (Reid, 1975), it is equally certain that apomorphine-induced effects also involve more than one central site.

A further hypothesis is that they also involve more than one type of dopamine receptor. As a preliminary division Kebabian and Calne (1979) have recently described D-1 and D-2 dopamine receptors, the former being linked to stimulation of a dopamine sensitive adenylyl cyclase enzyme (prototype receptor located in the bovine parathyroid gland) and the latter not being so linked (prototype receptor located in the mammotrophs of the anterior pituitary gland). Since apomorphine appears to be only a partial agonist or even an antagonist at D-1 sites, whereas it acts as a full agonist at D-2 sites, this division of dopamine receptors may also be used to explain some of the anomalous properties of the substituted benzamides which exhibit marked differences in potency as inhibitors of the dopamine sensitive adenylyl cyclase enzyme. However, although these differences tend to parallel the differences in potencies as displacers of radiolabelled dopamine receptor ligands the absolute potencies in the enzyme model are much less than those in the radiolabelled ligand model (Table 6). At the same time, the use of other ligands clearly demonstrates the selectivity of the substituted benzamides for dopamine receptors compared with α or β adrenergic receptors (Table 6).

TABLE 6 Comparison of the Potency of Clebopride, Metoclopramide and Sulpiride as Antagonists of DA-sensitive Adenylyl Cyclase and Displacers of Radiolabelled Ligands from their Specific Receptors.

Substituted benzamide	Inhib$^{n.}$ DA sensitive Ad.cyclase	[^{3}H]-Haloperidol striatum	[^{3}H]-Haloperidol limbic fb.	[^{3}H]-DHEC limbic fb.	[^{3}H]-DHAP limbic fb.
Clebopride	6.9×10^{-5}	8.4×10^{-8}	1.3×10^{-8}	2.2×10^{-5}	7.1×10^{-4}
Metoclopramide	8.0×10^{-4}	5.2×10^{-7}	5.2×10^{-7}	1.2×10^{-5}	2.5×10^{-4}
Sulpiride	1.3×10^{-3}	1.3×10^{-6}	2.0×10^{-6}	6.1×10^{-5}	2.8×10^{-3}

The Figures quoted are mean IC_{50} values (M) calculated from experiments performed with appropriate preparations of rat corpus striatum or limbic forebrain (nucleus accumbens, olfactory tubercle). DHEC, dihydroergocryptine; DHAP, dyhydroalprenolol. (Roberts and co-workers, 1978)

Of further importance is the possibility than the adenylyl cyclase linked D-1 receptors are predominantly post synaptic (intrastriatal injections of kainic acid cause a substantial loss of dopamine sensitive adenylylcyclase activity without reducing the content of dopamine) and that the non cyclase linked D-2 receptors are predominantly presynaptic (6-hydroxydopamine-induced nigral lesions do not alter nigral or striatal dopamine sensitive adenylyl cyclase activity), since their differential blockade could result in completely opposite biological effects. Thus, whereas a block of postsynaptic receptors will effectively inhibit the biological effects recognized as "dopaminergic", whether resulting from transmission of endogenous dopamine or the administration of exogenous dopaminergic drugs, a block of presynaptic receptors, by preventing the inhibitory effect of dopamine on synthesis and release, will result in increased dopaminergic transmission and potentiation of dopamine me-

diated effects. The pharmacological effects of a drug capable of blocking both receptors will obviously depend upon the relative degree of pre- and postsynaptic receptor blockade following any one dose or at any one time. Exogenous dopaminergic stimulants can also differentially affect these receptors. Apomorphine, for example, decreases locomotor activity in mice at low doses (due to stimulation of presynaptic receptors inhibiting the synthesis and release of dopamine) whereas at high doses it induces hyperactivity by a direct stimulant action on the postsynaptic receptors. These differential effects can be used to study the relative pre- and postsynaptic blocking activity of the substituted benzamides. Thus, both clebopride and sulpiride demonstrate presynaptic receptor blocking activity at low doses in that they inhibit the presynaptic apomorphine-induced decrease in locomotor activity (Berga, Beckett and Roberts, 1979) and, in common with sulmepride and prosulpride, potentiate the postsynaptic apomorphine-induced increase in locomotor activity (Puech, Simon and Boissier, 1978), in this latter case presumably due to the activity of the extra dopamine released adding to that of the apomorphine. At higher doses all of these compounds also show postsynaptic receptor blocking activity in that they inhibit the postsynaptic apomorphine-induced locomotor hyperactivity. By contrast, metoclopramide, sultopride and tiapride show no evidence of presynaptic receptor blocking activity although they readily block the postsynaptically mediated effects of apomorphine (Puech and co-workers, 1978; Berga and colleagues, 1979). These phenomena obviously provide an explanation of the different effects seen with the benzamides on apomorphine-induced hyperactivity shown in Table 3. Furthermore, Puech and co-workers (1978) have demonstrate a strong correlation between potentiation of apomorphine-induced hyperactivity and antagonism of apomorphine-induced hypothermia and have suggested that the latter effect is also presynaptically mediated. Certainly apomorphine does not induce hypothermia following degeneration of the dopaminergic fibres induced by 6-hydroxydopamine administration (Costentin and co-workers, 1974), whereas other effects persist, and such a concept is attractive as an explanation of the reversal of the antagonism of the apomorphine-induced hypothermia seen with sulpiride and similar compounds on increasing the dose (Table 3). Nevertheless, the observation that compounds such as metoclopramide, with little or no presynaptic receptor blocking activity, are also relatively potent antagonists of the hypothermic response to apomorphine indicates that postsynaptic interactions and/or non-dopaminergic mechanisms also contribute to the fall in body temperature.

Differential pre- and postsynaptic dopamine receptor blockade may also explain some of the therapeutic anomalies encountered with the substituted benzamide drugs. Thus cerebral dopaminergic malfunction has been implicated not only in schizophrenia and allied psychotic disorders (dopaminergic hyperactivity; Snyder and colleagues, 1974), but also in depression (dopaminergic hypoactivity; Randrup and Braestrup, 1977) and it is tempting to propose that the antidepressant activity of substituted benzamides such as sulpiride is related to a block of presynaptic dopamine receptors (resulting in increased release of dopamine) and that the neuroleptic activity seen with the same compounds at higher doses is related to a block of postsynaptic receptors.

Nevertheless, and in conclusion, it must be acknowledged that the arguements presented above are a gross oversimplification of reality since the dopaminergic systems are by no means autonomous and dopaminergic activity can be modified by drugs acting an other transmitter systems and viceversa. An understanding of such interactions, especially those with 5-hydroxytryptaminergic mechanisms (Leysen and Laduron, 1977;

Costall and Naylor, 1978; Roberts, 1979), will undoubtedly shed further light on the anomalous activities of the substituted benzamide drugs as dopamine receptor antagonists.

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Target Enzyme-Activated Irreversible Enzyme Inhibitors

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ABSTRACT

The concept of target enzyme-activated irreversible inhibition is introduced by describing inhibitors of the pyridoxal phosphate-dependent γ-aminobutyric acid transaminase (GABA-T) and ornithine decarboxylase (ODC). γ-vinyl GABA, an inactivator of GABA-T, is a substrate analogue which is activated by a mechanism involving enzymatically-induced prototropic shifts. α-acetylenic putrescine, an inactivator of ODC is a product analogue which was designed with the intention of exploiting the microscopic reversibility principle. The biochemistry of these inhibitors is discussed, followed by extrapolation of the concepts to the development of the first known inactivator of the NADPH-dependent steroid 5-α-reductase.

KEYWORDS

GABA-transaminase, γ-vinyl GABA, γ-acetylenic GABA, glutamic acid decarboxylase, ornithine decarboxylase, α-acetylenic putrescine, α-acetylenic ornithine, steroid 5-α-reductase, diazo ketones.

INTRODUCTION

An approach to the irreversible inactivation of enzymes is to design inhibitors that possess a latent reactive functionality which is unmasked at the enzyme's active site as a result of the normal catalytic turnover (Rando, 1974). This inhibitory process is described by the expression:

$$E + I \rightleftharpoons E \cdot I \xrightarrow[k_{cat}]{} E \cdot I^{*} \xrightarrow{k_{inhib}} E - I^{*}$$

where E = enzyme, I = inhibitor, I^{*} = transformed inhibitor.

I should hence be an extremely specific inhibitor because it should inactivate only those enzymes which are capable of processing it to I^{*}. Such known inhibitors are usually analogues of the normal enzyme substrate, however if one considers a typical enzyme-catalyzed reaction where a substrate S is converted to a product P:

$$E + S \rightleftharpoons E \cdot S \underset{k_{cat}}{\rightleftharpoons} E \cdot P \rightleftharpoons E + P$$

it becomes apparent that in view of the microscopic reversibility principle, I may also be an analogue of the product P.

If an inhibitor I acted by such a mechanism it could be expected to exhibit long-lasting biochemical and pharmacological effects as the enzyme should remain inactivated even after the free inhibitor is no longer present since regeneration of enzyme activity will be dependent on new enzyme synthesis. These dual attractions of specificity and extended duration of action have led us to explore the concepts of target enzyme-activated irreversible inhibition as an approach to drug design.

Owing to their well-understood mechanisms of action as well as their involvement in the biosynthesis and catabolism of a number of biogenic amines, the pyridoxal phosphate (PyCHO)-dependent enzymes initially suggested themselves as viable targets for attack by enzyme-activated irreversible inhibitors. Here we will discuss inactivators of some PyCHO-dependent enzymes as exemplified by 4-aminobutyrate:2-oxoglutarate aminotransferase (GABA-T) and L-ornithine decarboxylase (ODC). On the establishment of generic concepts for the irreversible inhibition of PyCHO-dependent enzymes, we will then turn to the inactivation of enzymes other than those which are dependent upon PyCHO, with an example of the inactivation of steroid 5-α-reductase, a NADPH-dependent enzyme responsible for the conversion of testosterone to the more active androgen, dihydrotestosterone.

INHIBITORS OF 4-AMINOBUTYRATE:2-OXOGLUTARATE AMINOTRANSFERASE (GABA-T)

The recognition of 4-aminobutyric acid (GABA) as an important inhibitory neurotransmitter in mammalian brain (Krnjevic, 1974) has stimulated the search for inactivators of GABA-T. GABA-T is the GABA catabolizing enzyme and its inactivation could be expected to potentiate GABA neurotransmission and hence may be useful in the treatment of diseases where a deficiency of GABA function has been demonstrated or implicated, e.g. Huntington's disease (Bird and Iverson, 1974) and schizophrenia (Roberts, 1974).

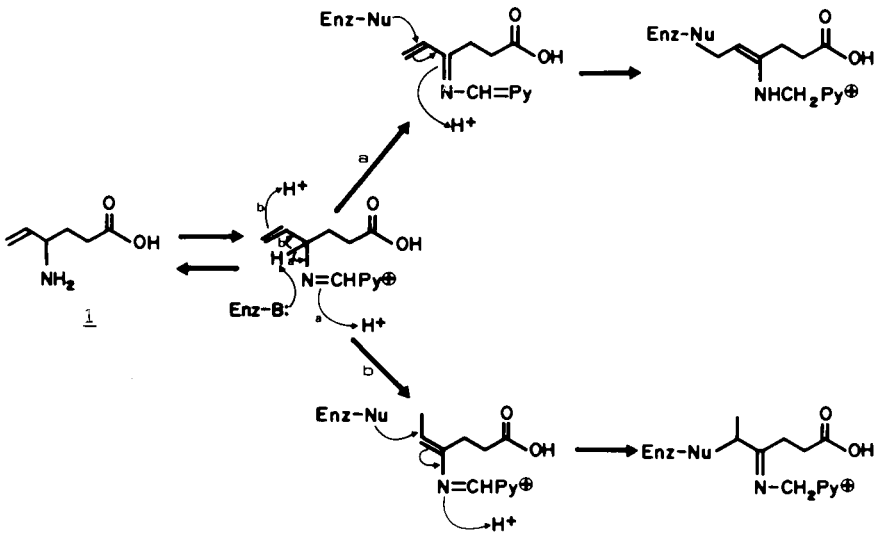


Fig. 1. Inhibition of GABA-T by γ-vinyl GABA (1).

GABA-T, being a PyCHO-dependent enzyme, operates via a tautomerism of the Schiff base formed between GABA and PyCHO. The Schiff base function activates the adjacent C-H bond so that proton abstraction is facilitated. Reprotonation leads to the tautomeric Schiff base which is hydrolyzed to the products, succinic semialdehyde and pyridoxamine. We hoped that the synthetic GABA analogue, γ-vinyl GABA (4-aminohex-5-enoic acid, 1) would be accepted as a substrate in the same manner as GABA itself (Fig. 1). Thus the normal transamination reaction (Path a) would convert the vinyl group which in 1 is an unreactive appendage, to an active alkylating function which could react with a nucleophilic residue (Nu) in the active site leading to covalent bond formation and hence irreversible inactivation. Alternatively, (Path b) isomerism of the double bond would generate a new double bond, which would be conjugated through to the pyridine ring and hence could also be capable of eliciting irreversible inactivation by covalent attachment to the active site. The transamination pathway (Path a) is the one that has been found by Rando, Relyea and Cheng (1976) to be operative in the irreversible inhibition of aspartate transaminase by 2-amino-4-methoxy-trans-3-butenoic acid. On the other hand, vinylglycine inhibits the same enzyme via the isomerism pathway (Gehring, Rando, and Christen, 1977).

When GABA-T, partially purified from rat brain is incubated for varying time periods with γ-vinyl GABA, then assayed for remaining enzyme activity, a time-dependent inactivation process is observed which follows pseudo first order kinetics (Lippert and co-workers, 1977). Enzymatic half lives range from 11 min to 1 min with concentrations of inhibitor between 0.05 mM and 1 mM. If GABA or glutamate is added to the incubation medium the rate of inactivation induced by γ-vinyl GABA is dramatically reduced demonstrating that the inactivation process is active-site directed. Evidently the normal substrates GABA and glutamate convert the holoenzyme to the pyridoxamine form which cannot bind the inhibitor as Schiff base formation is no longer possible. The detection of a kinetic isotope effect on the rate of inhibition using 4-deuterio-4-aminohex-5-enoic acid demonstrates that the inactivation process involves cleavage of the γ C-H bond and confirms that γ-vinyl GABA must be a substrate in order for inactivation to occur.

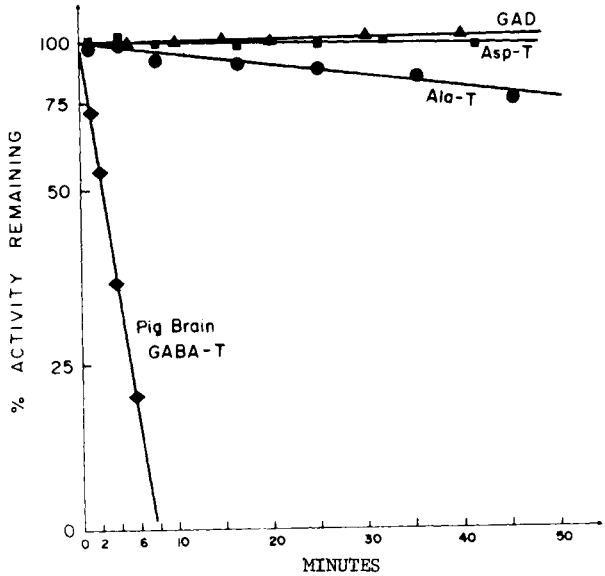
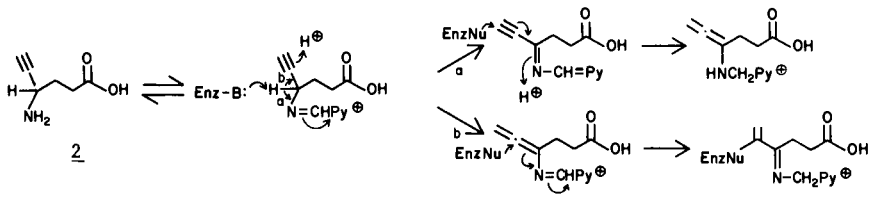


Fig. 2. The effect of γ-vinyl GABA on rat brain glutamate decarboxylase, aspartate transaminase, alanine transaminase and GABA-T.

As anticipated, γ-vinyl GABA proves to be an extremely specific inhibitor of mammalian GABA-T. Figure 2 (Lippert and co-workers, 1977) compares the rate of inactivation of GABA-T induced by 0.5 mM concentration of inhibitor with its effects at 10 mM on glutamate decarboxylase, aspartate transaminase and alanine transaminase, all of which are PyCHO-dependent. While there is no effect on the first two enzymes, there is a slow inactivation of rat brain alanine transaminase that is about 1000 times slower than that of GABA-T (Lippert and co-workers, 1977).

When GABA-T, which has been inactivated by γ-vinyl GABA is subjected to exhaustive dialysis for 4 days at pH values ranging from 5-9 with buffers containing PyCHO only 5-10% of control activity is restored, thus demonstrating the irreversibility of the process. This irreversibility is reflected in the long duration of action when the inhibitor is administered to mice. A single dose of 1000 mg/kg i.p. produces a rapid decrease in brain GABA-T activity to approximately 25% of control, this level of inhibition being maintained for at least 48 hours. Even after 5 days GABA-T is only at 75% of control. Brain GABA levels increase to five to sixfold over control, this level persisting for over two days (Jung and co-workers, 1977b).

Path a in Fig. 1 suggests that if the vinyl group were to be replaced by an acetylene group ie. if γ-acetylenic GABA (4-aminohex-5-ynoic acid 2) could be accepted as a substrate by GABA-T, then inactivation could also ensue via the transamination pathway (Path a, Fig. 3). Alternatively, (Path b) prototropic rearrangement could



2

Fig. 3. Inhibition of GABA-T by γ-acetylenic GABA (2).

lead to a conjugated allene which could once again elicit inactivation. In practice, 2 does inactivate GABA-T in vitro (Jung and Metcalf, 1975), and proves to be more active (Jung and co-workers, 1977a) than γ-vinyl GABA (1) in vivo. Further studies revealed that 2 also inactivates glutamic acid decarboxylase (GAD), but to a lesser extent than GABA-T. As γ-acetylenic GABA (2) is an analogue of GABA, the product of decarboxylation of glutamic acid catalyzed by GAD, its inactivation of GAD appeared to be a consequence of the microscopic reversibility principle, and we decided to verify that this was the case. Commercially-available bacterial GAD was chosen for in vitro study since the stereochemistry of the enzymatic replacement of carboxyl by hydrogen had been determined and been found to proceed with retention of configuration (Yamada and O'Leary, 1978).

Incubation of GAD from E. coli with 2 results in a time-dependent loss of enzyme activity which follows pseudo first order kinetics until the inhibition is essentially complete. Protection against inactivation is afforded by 2-methylglutamate and by L-glutamate itself demonstrating that the inactivation process is active site directed. A kinetic isotope effect of 2.5 at 0.33 mM inhibitor is found when the rate of inhibition induced by 4-deuterio-4-aminohex-5-ynoic acid is compared with that observed with 2 demonstrating that inhibition involves a catalytically-functioning enzyme and requires abstraction of the propargylic hydrogen. The inhibition is also stereospecific as (±) 2 was resolved and the inhibitory activity found to reside with the (-) isomer. This was assigned the R absolute configuration as oxidation with RuO_4 afforded R(-)glutamate, while the (+) isomer yielded

S(+)glutamic acid (Jung and co-workers, 1978). These results are compatible with a mechanism of inactivation which relies on the microscopic reversibility principle. As the decarboxylation reaction occurs with retention of configuration at the α-carbon, (Yamada and O'Leary, 1978) it is the pro-4(R)-hydrogen of GABA, which is potentially labile in the reverse direction. If 4(R)-4-aminohex-5-ynoic acid, which bears a proton stereochemically corresponding to that which replaces CO_2 in 2(S)glutamic acid, can replace GABA in the active site, then the proton abstraction implicit in the reverse reaction should lead to the formation of a propargylic anionic intermediate which could induce irreversible inactivation of the enzyme by analogy to either paths a or b, Fig. 3. Although proton exchange of the 4-pro(R)-hydrogen of GABA catalyzed by GAD has not been detectable, (Yamada and O'Leary, 1978) it is feasible that with 4(R)-4-aminohex-5-ynoic acid, this proton abstraction is facilitated by the adjacent acetylene group.

GABA-T has also proven susceptible to inactivation by other enzyme-activated irreversible inhibitors (Metcalf, 1979). Among these are gabaculine (Rando and Bangerter, 1976) and isogabaculine (Metcalf and Jung, 1979) which inhibit via the conversion of a cyclohexadienylamino acid to an aromatic species which binds tightly to the active site, while ethanolamine-O-sulfate leads to generation of an alkylating species in the active site by a β-elimination of sulfate (Fowler and John, 1972). The actions of these compounds illustrate a number of mechanism types which have been further exploited in the inhibition of other PyCHO-dependent enzymes. In the next section, we will discuss the irreversible inactivation of ornithine decarboxylase by a product analogue, designed to take advantage of the microscopic reversibility principle.

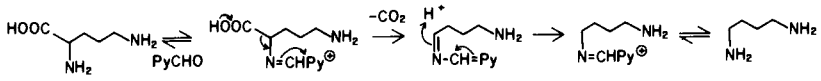
INHIBITORS OF ORNITHINE DECARBOXYLASE (ODC)

This PyCHO-dependent enzyme catalyzes the decarboxylation of ornithine to the diamine putrescine. Putrescine is then converted to the higher polyamines spermidine and spermine (Russell, 1973). These polyamines have been implicated in the regulation of growth processes and an induction of ODC, with the resultant elevation of polyamine levels, has been correlated with conditions of rapid cell proliferation (Mamont and co-workers, 1976).

The mechanism for the decarboxylation of ornithine to putrescine is shown in Fig. 4 and is representative of all α-amino acid decarboxylases. Ornithine, via its α-amino group forms a Schiff base with PyCHO, loss of CO_2 then generates a Schiff base-stabilized carbanion which is reprotonated and the resulting Schiff base hydrolyzed to putrescine and PyCHO.

With the intention of exploiting the microscopic reversibility principle, the putrescine analogue, α-acetylenic putrescine (5-hexyne-1,4-diamine, 3) was synthesized (Metcalf and co-workers, 1978a). It was anticipated that if α-acetylenic putrescine (3) could replace putrescine in the active site than the proton abstraction implicit in the reverse reaction should lead to the formation of a propargylic anion intermediate which could induce irreversible inactivation by either of the paths a or b (Fig. 4).

Ornithine decarboxylase



Proposed inhibition by α-acetylenic putrescine

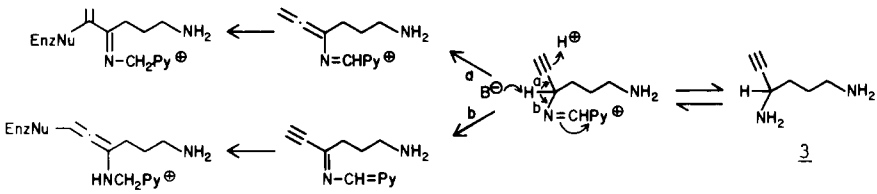


Fig. 4. Inactivation of ornithine decarboxylase by α-acetylenic putrescine (3).

Incubation of the enzyme preparation obtained from livers of thioacetamide-treated rats at pH 7 with 3 resulted in a time-dependent loss of enzyme activity which followed pseudo first order kinetics for at least two half lives (Fig. 5). Over longer time periods, the semilogarithmic plots deviated from linearity. However, incubation with 3 at 0.1 mM concentration resulted in 95% inactivation of ODC after 10 min. Prolonged (24 h) dialysis of enzyme previously inactivated by 3 against a buffer solution containing phosphate (30 mM), pyridoxal phosphate (0.1 mM), and dithiothreitol (5 mM) (conditions where the native enzyme is stable) did not lead to regeneration of enzyme activity, thus demonstrating the irreversibility of the process. That the inhibition of ODC is active site-directed is shown by the

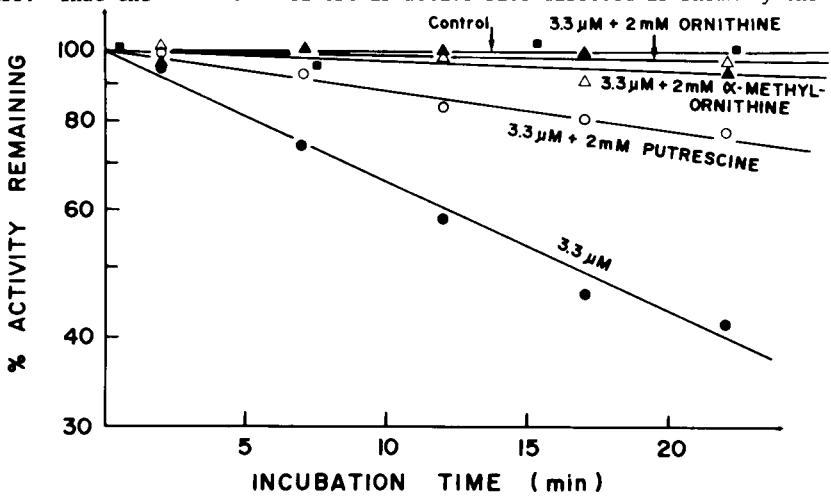


Fig. 5. Inactivation of rat liver ODC by (±)-α-acetylenic putrescine (3).

protective effects of the natural substrate L-ornithine, of a competitive inhibitor 2-methylornithine and of putrescine, the product of decarboxylation, against induced inactivation (Fig. 5). The presence of dithiothreitol (5 mM) in the preincubation medium and the absence of lag time before the onset of inhibition rule out the possibility of inhibition via an affinity labeling mode by a diffusible alkylating species (Metcalf and co-workers, 1978a).

3 inhibits ODC *in vivo*, with a single dose of 100 mg/kg i.p. in rats producing a near-total decrease of ornithine decarboxylase activity in prostate and to a lesser extent in thymus and testis. Three doses of 100 mg/kg of 3 during a 24 hour period markedly decreased putrescine concentrations in the three organs studied, while spermidine levels were also lowered in the prostate (Danzin and co-workers, 1979).

Prolonged treatment of rats with 3 induces behavior reminiscent of that of animals which had received the GABA-T inhibitor γ-acetylenic GABA (Jung and co-workers, 1977a). It has now been confirmed that 3 is converted to γ-acetylenic GABA (2) via a mitochondrial pathway involving monoamine oxidase (Danzin and co-workers, 1979). This unwanted transformation has been overcome with the incorporation of a δ-methyl group in 3, the substituted analogue being no longer a substrate for monoamine oxidase. ODC inhibitory activity, however, is retained (Casara and co-workers, 1980).

ODC is also subject to inactivation by a substrate analogue bearing an α-acetylenic group. As shown in Fig. 6, if α-acetylenic ornithine (4) is accepted as a substrate, enzyme-induced loss of CO_2 would generate the same propargylic carbanion intermediate proposed in the inhibition of ODC by the product analogue, α-acetylenic putrescine (3). Covalent attachment could then ensue via either of paths a or b. In practice, α-acetylenic ornithine (4) does inactivate ODC with an apparent dissociation constant (K_I) of 10 μM and T 1/2 (half life at infinite concentration) of 8.5 minutes (Danzin and co-workers, 1980). This is to be compared with a K_I of 2.3 μM and T 1/2 of 9.7 minutes for α-acetylenic putrescine (3).

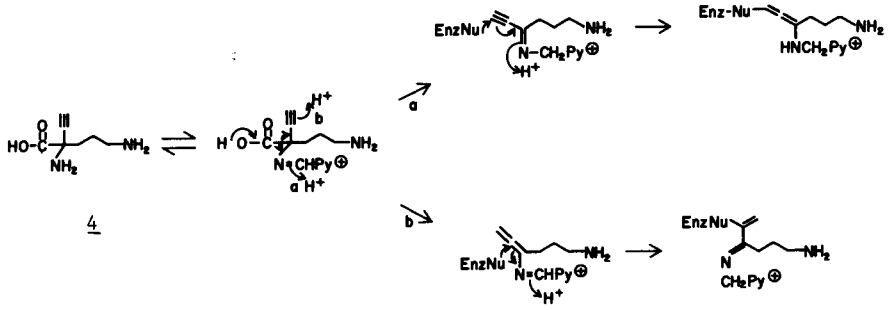


Fig. 6. Inactivation of ODC by α-acetylenic ornithine. (4).

Bey and co-workers (1978) have demonstrated that amino acid decarboxylases may also be inactivated by fluorinated derivatives of the natural substrate. Thus, α-difluoromethylornithine (5) has been found to be an irreversible inactivator of ODC, the proposed mechanism (Fig. 7) involving a decarboxylative elimination of fluoride to generate a Michael acceptor in the active site (Bey, 1978; Metcalf and co-workers, 1978a).

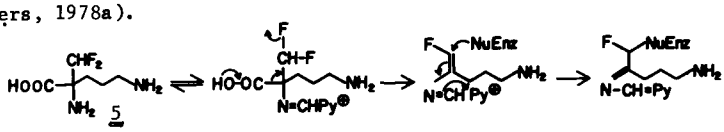


Fig. 7. Inactivation of ODC by α-difluoromethyl ornithine (5).

Other PyCHO-dependent enzymes have been found to be susceptible to inhibition by acetylenic or fluoromethyl analogues of the normal substrate or product of the enzymatic reaction. β-Fluoroalanine inactivates bacterial alanine racemase (Wang and Walsh, 1978), β,β,β-trifluoroalanine and propargyl glycine inactivate γ-cystathionase (Silverman and Abeles, 1977), while a number of monofluoromethyl amines and aminoacids irreversibly inhibit the corresponding amino acid decarboxylases (Kollonitsch and co-workers, 1978; Jung and co-workers, 1979).

At the moment, it is difficult to predict for a given decarboxylase, whether the substrate or product analogue, either acetylenic or mono, di- or tri-fluoromethyl, will prove to be a more effective inhibitor. Such answers will await the synthesis and testing of all the possibilities.

With the establishment of generic approaches to the inactivation of PyCHO-dependent enzymes by acetylenic and fluoromethyl analogues we decided to attempt to extend those concepts to the inhibition of enzymes other than those in the PyCHO class, and steroid 5-α-reductase, to be discussed in the next section, appeared to be an appropriate target.

APPROACHES TO THE INHIBITION OF STEROID 5-α-REDUCTASE

The NADPH-dependent enzyme steroid 5-α-reductase catalyzes the reduction of 3-keto-Δ-4,5-steroids to the corresponding 5-α-dihydro analogues and is of physiological importance in the conversion of testosterone to the target tissue active androgen, 5-α-dihydrotestosterone (Bruchovsky and Wilson, 1968). Inhibitors of steroid 5-α-reductase hence may offer a means to control excessive manifestation of androgenic action in diseases such as acne (Sansone and Reisner, 1971) and benign prostatic hyperplasia (Siiteri and Wilson, 1970).

A plausible mechanism for the NADPH-dependent reduction of testosterone to 5-α-dihydrotestosterone is an initial priming of the enone system by protonation with donation of hydride to the 5-α-position. The resultant enol then tautomerizes to the ketone (Fig. 8). In an initial attempt to inactivate this enzyme we hoped, by analogy with the inhibition of PyCHO-dependent enzymes by acetylenic and fluoromethyl analogues, that 4-ethynylandrost-4-ene-3,17-dione (6) or 4-fluoromethyl testosterone (7) would inactivate steroid 5-α-reductase by mechanisms shown in Fig. 9. Thus, in each case hydride addition should lead to an enol which in the

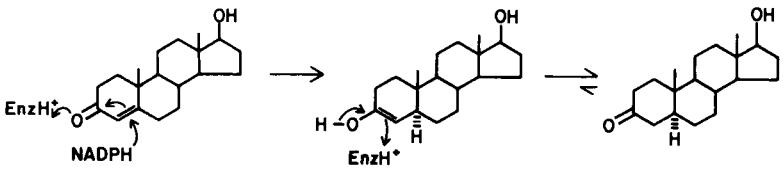


Fig. 8. Steroid 5-α-reductase

case of 6 could rearrange to the conjugated allene, a potential alkylating agent, or with 7 to β-elimination of fluoride to generate a highly-active exomethylene ketone. We synthesized both 6, a known compound (Julia and Moutonnier, 1964) and 7, only to find that neither induced a time-dependent inactivation of steroid 5-α-reductase (Metcalf and co-workers, 1978b).

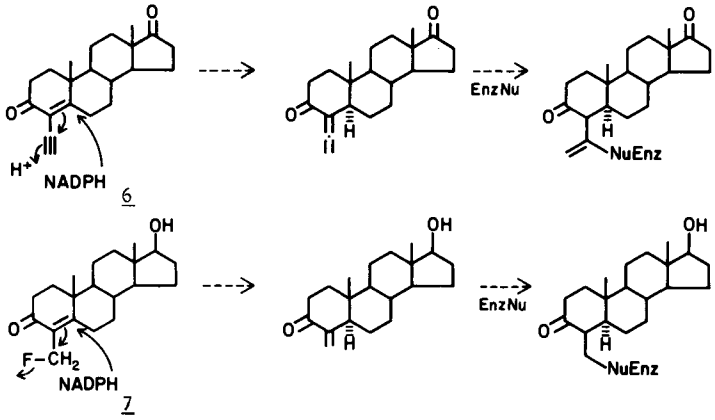


Fig. 9. Proposed mechanism of inactivation of steroid 5-α-reductase by 4-acetylenic and 4-fluoromethyl steroids.

The mechanisms proposed in Fig. 9 have, however, ignored the potential of the enzyme to protonate either the 3-keto function or carbon 4. By taking advantage of this protonation ability, we hoped that a diazoketone analogue of the substrate would induce irreversible inactivation. This use of diazoketones is suggested by the irreversible inhibition of N-formylglycinamide ribonucleotide (FGAR) amidotransferase by the naturally-occurring diazoester, azaserine (Buchanan, 1978). As part of its normal mechanism of action (Fig. 10) FGAR amidotransferase protonates the amido group of the natural substrate glutamine, thereby facilitating nucleophilic displacement by an enzymatic sulfhydryl group to generate enzyme-bound ammonia and glutamate. Buchanan (1978) has demonstrated that azaserine, a glutamine analogue is protonated by FGAR amidotransferase and the resultant diazonium ion then alkylates the active site sulfhydryl group resulting in inactivation of the enzyme (Fig. 10).

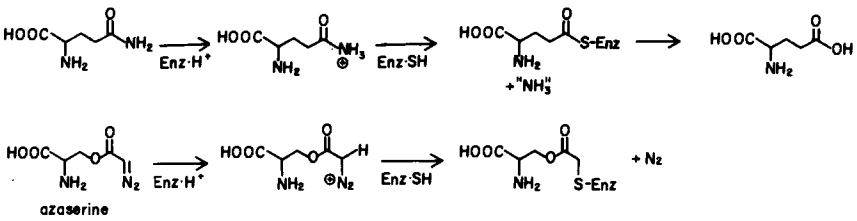
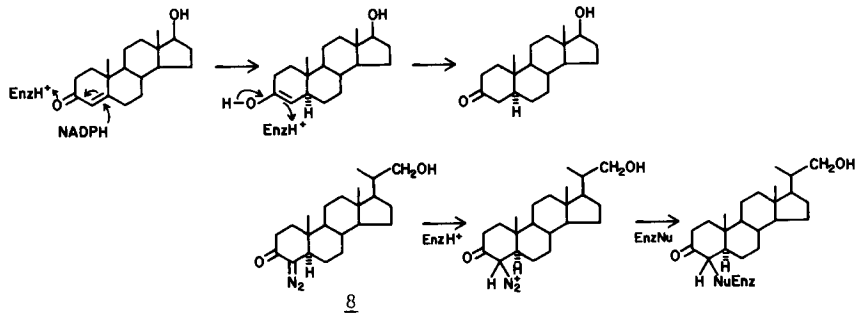


Fig. 10. Inactivation of FGAR amidotransferase by azaserine.

Based on this analogy we hoped that the 3-keto-4-diazo-5-α-dihydrosteroid 8 would prove to be a specific irreversible inhibitor of steroid 5-α-reductase. Thus, if the diazo ketone 8 binds to the active site, the ability of the enzyme to protonate at carbon 4 should lead to a reactive diazonium species which could be alkylated by a nucleophilic residue in the active site, leading to irreversible inhibition (Fig. 11).



8

Fig. 11. Inactivation of steroid 5-α-reductase by a 3-keto-4-diazo steroid.

The analogue 8 with a hydroxy function at C_{22} was chosen as a target compound rather than the 5-α-dihydrotestosterone analogue as it has been shown that modifications of the side chain at 17 can lead to compounds with a higher affinity for the active site than testosterone itself (Voight and Hsia, 1973; Benson and Blohm, 1978). When the diazoketone 8 (Metcalf, Jund and Burkhart, 1980) was incubated with a microsomal preparation containing steroid 5-α-reductase from rat prostate in the presence of an NADPH generating system, and aliquots were removed at various time intervals and assayed for 5-α-reductase activity, a time-dependent decrease in enzyme activity was observed (Fig. 12, Blohm and co-workers, 1980). The decline in enzyme activity followed first order kinetics for two half lives and on analyzing the data according to Jung and Metcalf (1975), where the enzyme half life is plotted against the inverse of the inhibitor concentration, the apparent dissociation constant K_I was found to be 3.5×10^{-8} M, and the T 1/2 16.5 minutes. The observed inhibition at zero time is apparently due to a competitive component of inhibition.

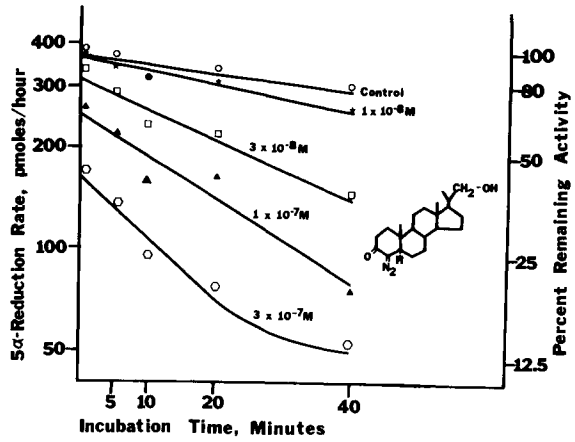


Fig. 12. Inactivation of steroid 5-α-reductase by 8.

When the substrate, testosterone is added to the incubation mixture, the rate of inactivation induced by the diazoketone 8 is slowed down indicating that the inhibition process is active site directed. Owing to the instability of steroid 5-α-reductase it was not possible to utilize exhaustive dialysis as a means of confirming the irreversibility of inhibition and more definitive experiments will await availability of a labeled inhibitor. A time-dependent inactivation process, however, is usually taken as strong indication of irreversible enzyme inactivation (Abeles and Maycock, 1976).

As far as we can tell, the inactivation of prostatic steroid 5-α-reductase by the diazoketone 8 is a specific process in that aromatase, Δ^5-3-keto isomerase, 3-α-hydroxy steroid oxidoreductase and steroid 5-β-reductase which process testosterone or 5-α-dihydrotestosterone (Fig. 13) are unaffected in vitro at concentrations up to 10^{-6} M (Blohm, Johnston and Wiseman, 1980).

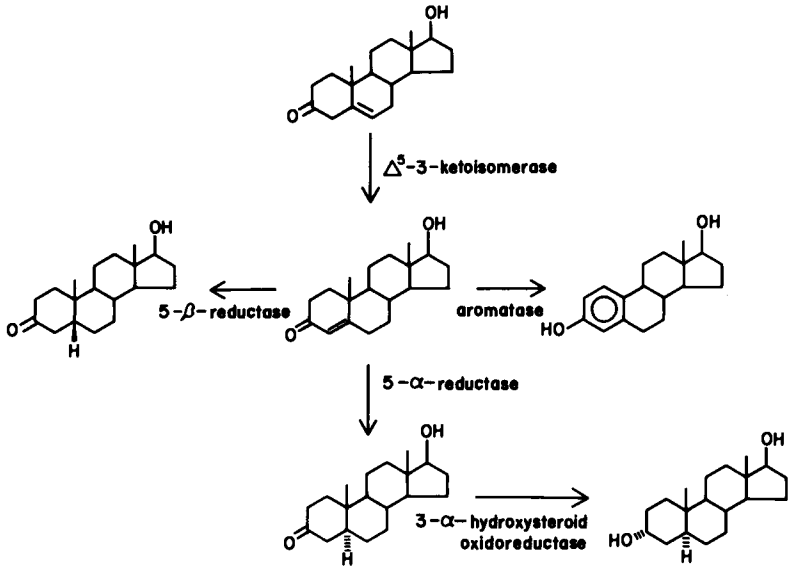


Fig. 13. Some enzymes which possess testosterone.

When administered to rats intragastrically at 100 mg/kg, 8 produced a reduction of prostatic 5-α-reductase activity of 60 percent, which was maintained for at least 8 hours (Blohm and co-workers, 1980). The specificity, apparent irreversibility, and oral activity of the diazoketone 8 suggest that this inhibitor will be a valuable tool in the elucidation of the physiological roles of 5-α-reduced androgens.

PERSPECTIVE

Irreversible inactivators of the PyCHO-dependent enzymes which catalyze the formation or destruction of most of the aminergic neurotransmitter substances have now been reported (Jung, Koch-Weser and Sjoerdsma, 1980; Kollonitsch and co-workers, 1978) and some of these are undergoing pharmaceutical development with the ultimate aim of demonstrating therapeutic usefulness in man. In this connection we may note that GABA-T inhibitors, represented by γ-vinyl GABA have anticonvulsant effects in animals (Schechter and co-workers, 1977) including primates (Meldrum, 1978), while

α-difluoromethyl ornithine, a representative inhibitor of ODC has a demonstrated antigestational property (Fozard and co-workers, 1980), antitrypanosomal activity (Bachi and co-workers, 1980) and antitumor effects (Prakash and co-workers, 1980) in animals.

The stage is now set for the exploitation of the concept of enzyme-activated inhibition to enzymes which do not depend on PyCHO. A number of examples already exist including Bloch's classic study on the inactivation of β-hydroxydecanoyl thioester dehydrase by acetylenic thioesters (Endo, Helmkamp and Bloch, 1970), clinically useful inactivators of thymidylate synthetase (Santi, 1980) and a host of monoamine oxidase inhibitors (Knoll, 1978; Sandler, 1978), thus demonstrating the potential general applicability. The design of enzyme-activated irreversible inhibitors is of course not the sole approach to enzyme inhibition, but should be considered complementary to approaches based on transition state theory or judicial utilization of binding sites. In the search for the rational design of therapeutically-useful substances it is, however, a powerful new option.

ACKNOWLEDGMENTS

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Figure 1 is reproduced from B. W. Metcalf and co-workers (1978) in "Enzyme-Activated Irreversible Inhibitors", N. Seiler, M. J. Jung and J. Koch-Weser, eds. (Elsevier/North-Holland, Amsterdam) 27-41; Fig. 2 from B. Lippert and co-workers (1977), "4-Aminohex-5-enoic Acid, a Selective Catalytic Inhibitor of 4-Aminobutyric Acid Aminotransferase in Mammalian Brain", Eur. J. Biochem., 74, 441-445; Fig. 5 from B. W. Metcalf and A. Sjoerdsma (1979) in "Drug Action and Design: Mechanism Based Enzyme Inhibitors", T. A. Kalman, ed., (Elsevier/North-Holland Inc.), 61-73; Fig. 10 from B. W. Metcalf, K. Jund and J. Burkhart (1980), "Synthesis of 3-keto-4-diazo-5-α-Dihydrosteroids as Potential Irreversible Inhibitors of Steroid 5-α-Reductase", Tetrahedron Letters, 15-18 (Pergamon Press Ltd.); Fig. 12 from T. Blohm and co-workers (1980), "Inhibition of Testosterone 5-α-reductase by a Proposed Enzyme-Activated, Active Site-Directed Inhibitor", Biochem. Biophys. Res. Commun., 95, 273-280 (Academic Press, Inc.). All reproductions are with permission of the respective copyright holder.

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Proteolytic Enzyme Inhibitors

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ABSTRACT

A review on the various types of proteinase inhibitors with particular emphasis on their differences in molecular properties and reaction mechanisms is given.

KEYWORDS

Proteinase Inhibitors, Microorganisms, Blood Plasma, α_2-Macroglobulin, Collagenase, Latent Enzyme, Reaction Mechanism, Thiol Disulfide Exchange, Enzyme-Inhibitor Association.

INTRODUCTION

Control of proteolytic enzymic activity is fundamental for a wide variety of important biological processes, e.g. blood clotting and clot lysis, pressure regulation, hormone and kinine liberation, protein turnover, assembly and processing, fertilization, phagocytosis, complement immune reactions and many others, see reviews (1-11). Generally the physiological function of the proteinase inhibitors is inactivation of untimely or locally unwanted proteolytic activity. Their primary interaction with the enzyme is a substrate like association at the substrate binding region. A classification of the proteinase inhibitors may be accomplished by inhibiting one of the four mechanistic classes of proteinases (12): carboxyl, metallo, sulfhydryl or serine proteinases, or may be accomplished by their differing mechanisms of reaction with the target enzyme or their different biological sources. All classifications suffer from the fact that only the large group of low molecular weight protein proteinase inhibitors of serine proteinases has so far been reasonably well investigated.

It is the aim of this review to emphasize on the differences in general properties and reaction mechanism of the various types of inhibitors. Some types are only found in certain living organism, e.g. the microbial peptide inhibitors in microorganisms or α_2-macroglobulin in the plasma of mammels. Some representatives will be chosen since an almost complete list with summary of their inhibitory activities is far beyond the scope of this article. Enzyme-activated inhibitors (synthetic chemical compounds, e.g. suicide substrates) will not be discussed here.

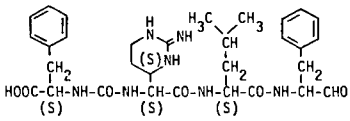
MICROBIAL INHIBITORS

In the last decade several proteinase inhibitors from microbial origin have been isolated and characterized according to their inhibitory properties, Table 1, for reviews see(13 - 15).

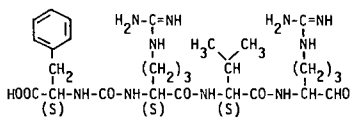
TABLE 1 Microbial Inhibitors

Inhibitor	Species	Proteinase inhibited
Leupeptin	Streptomyces	Thrombokinase, Plasmin, Trypsin, Papain, Kallikrein, Cathepsin B
Antipain	"	Papain, Trypsin, Thrombokinase, Plasmin
Chymostatin	"	Chymotrypsin, Papain, Cathepsin A, B und D
Elastatinal	"	Elastase
Pepstatin	"	Pepsin, Cathepsin D
Amastatin	"	Aminopeptidase A
Bestatin	"	Aminopeptidase B, Leucine aminopeptidase
Elasnin	"	Elastase
API-2b	"	Alkaline Protease, Subtilisin BPN u. Carlsberg Pronase S
Phosphor-amidon	B.thermolyticus	Thermolysin
E-64	Aspergillus	Papain, Ficin, Bromelain

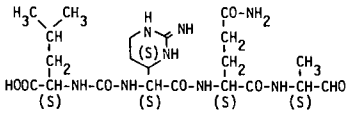
Leupeptin strongly inhibits the thrombokinase reaction, fibrinogenolyse and fibrinolyse by plasmin, and the proteolysis of casein and arginine amide substrates by trypsin and papain. The hydrolysis of benzoyl arginine ethyl ester by glandular kallikrein is also inhibited. Antipain is a more specific inhibitor of papain and trypsin compared to leupeptin, but inhibits cathepsin B, a trypsin-like tissue proteinase. Furthermore antipain inhibits the activity of cathepsin A, a carboxylpeptidase, which is not inhibited by leupeptins. However, the antiplasmin activity measured by the inhibition of fibrinolysis is much weaker than that of leupeptin. Chymostatin shows strong inhibition towards α, β, γ and δ-chymotrypsin and papain, but rather small effects on plasmin, thrombin, thrombokinase, kallikrein and pepsin. Elastatinal is a specific inhibitor of pancreas-elastase.



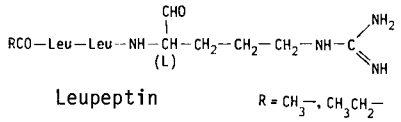
Chymostatin



Antipain



Elastatinal



Leupeptin

Fig. 1

The inhibitors leupeptin, chymostatin, elastatinal and antipain are tetrapeptide derivatives with an aldehyde group which is essential for inhibitory activity. Their structures are given in Fig. 1. The inhibition reaction depends on the aldehyde group. The corresponding ketoderivates Ac-leu-leu-phenethylamine and Ac-leu-leu-amidophenylbutanone of the synthetic inhibitor Ac-leu-leu-phenylalaninal are without effect on the caseinolysis by chymotrypsin (16). The formation of a tetrahedral intermediate (acetale) as a transition state analog is suggested to be important for the inhibitory activity. The enzymes attacking nucleophile seems capable of forming such stable intermediate at the aldehyde carbon atom, similar to the intermediate proposed for the reaction of certain hydrolases with ester substrates. The general mechanism of proteolysis (hydrolysis) involving enzyme acylation and deacylation proposes the tetrahedral intermediate in the formation of the acylenzyme. Thus substrates capable of forming tetrahedral adducts during the acylation and deacylation steps might be expected to exhibit an unusual affinity for these enzymes. The thiol proteinase inhibitor E-64 from Aspergillus japonicus specifically inhibiting papain and cathepsin B (17) contains a trans-epoxysuccinic acid moiety (18) which might be involved in a covalent binding to the thiolproteinase by an addition reaction to the enzymes nucleophile together with an opening of the epoxy ring.

Pepstatin is a non-aldehyde inhibitor of the acidic carboxyl proteinase pepsin, renin and of gastricin, Fig. 2. The inhibitory activity depends on the concentration of pepsin. The hydrophobic part in the structure of pepstatin and the hydroxyl group of the 4-amino-3-hydroxy-6-methylheptanoic acid moiety is thought to play an important role in the binding of pepstatin to pepsin in that a tetrahedral transition state is mimicked by the hydroxyl group at the scissile peptide carbonyl. The interaction is not mediated by a covalent bond as seems possible with the aldehyde inhibitors.

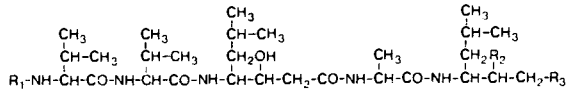
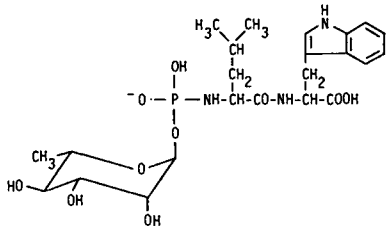
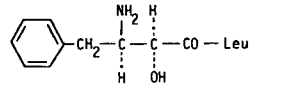


Fig. 2. Pepstatin: general structure with R_1=valeryl, caproyl, heptanoyl or capryl, R_2=O or HOH and R_3=COOH or H.

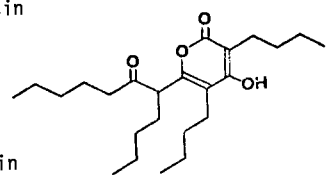
Similar binding could also apply to Amastatin, (2S,3R)-3-amino-2-hydroxy-5-methylhexanoyl—Val-Val-Asp (19), that inhibits aminopeptidase A and to Bestatin, an inhibitor of aminopeptidase B (2o) as well as to the non-peptide compound elasnin, a novel inhibitor of elastase (21). A chelating effect with the central Zn^{++} atom in the metalloenzym thermolysin was proposed for binding to the inhibitor phosphoramidon isolated from Actinomycetes (22).



Phosphoramidon



Bestatin



Elasnin

Fig. 3

PLASMA α_2-MACROGLOBULIN

Human plasma contains several proteins which inactivate proteinases, Table 2, for reviews see (1-9). With the exception of α_2-macroglobulin (α_2M), all inhibitors form 1:1 stochiometric and enzymically inactive complexes with the enzymes (see below). α_2M differs from all other proteinase inhibitors in many respects, for review chapters see in (1-9).

TABLE 2 Survey of the Protease Inhibitors from Human Plasma

Name	Concentration (mg/1oo ml)	Mr	Amino Acid Content %	Carbohydrate Contend %	Chain Composition (Number)
α_1-antitrypsin	29o ± 45	54,ooo	86	12	1
α_1-antichymotrypsin	49 ± 7	69,ooo	73	25	1
inter-α-trypsin inhibitor	5o	16o,ooo	9o	8	
antithrombin III	24 ± 2	65,ooo	85	13	1
$\overline{C_1}$-inactivator	24 ± 3	1o4,ooo	65	35	1
α_2-antiplasmin	7 ± 1	7o,ooo	87	13	1
inhibitor of plasminogen activation		8o,ooo			
α_2-macroglobulin	26o ± 7o	725,ooo	92	8	4

α_2M is a tetrameric glycoprotein with a molecular weight of 72o.ooo built of four immunologically and electrophoretically identical polypeptide chains. The four subunits are linked in pairs by disulfide bonds and the dimers are associated by noncovalent interactions dissoziable upon denaturation. The characteristics of the interaction with proteinases are: 1. Only active endopeptidases react; 2. Almost all enzymes are irreversibly bound; 3. Presaturation of α_2M with one proteinase prevents the subsequent binding of another; 4. The substrate reactions of the bound enzyme are sterically hindered.

α_2M reacts with virtually all four mechanistic classes of active endopeptidases, i.e. serine, thiol, carboxyl and metal proteinases, from a wide variety of bacterial, plant and animal sources including some of very limited specifity such as mammalian collagenases (23). Exopeptidases, zymogens and some highly specifity endopeptidases such as Hageman factor and urokinase do not react (24, 25). Most published data report that either one or two molecules of proteinase are bound per molecule α_2M (26). The rates of reaction vary considerably. Most enzymes are irreversibly bound. Radioactively-labeled proteinase bound to α_2M was reported not to be displaced by unlabeled molecules of the same or another proteinase (27), however, a snake proteinase is released from the complex (28). Interaction between α_2M and proteolytic enzymes with which complexes are formed is accompanied by proteolytic cleavage of the 185,ooo molecular weight subunit structure. Trypsin, plasmin, thrombin, plasma kallikrein and chymotrypsin produced a 85,ooo molecular weight derivative polypeptide chain (chymotrypsin as well 9o,ooo and 75,ooo molecular weight) (26). Experimental evidence was obtained that covalent bond formation accompanies the reaction of the proteinase with α_2M (26). This explains the irreversible binding of the enzyme and does not support the entrapment hypothesis from Barret and Starkey (29). This hypothesis suggested that following cleavage of the peptide bond the α_2M entrappes the enzyme with α_2M being the enveloping inhibitor molecule. A structural change of the enzyme as an α_2M-induced transconformation

has been reported and may account for the change of the enzyme's kinetic parameters upon binding (3o). Reactions of the α_2M bound enzyme with macromolecular substrates, protein inhibitors, and antibodies appear to be sterically hindered, but reactions with diisopropyl phosphorofluoridate and small synthetic substrates occur with 8o% - 1oo% of its normal activity. Electron microscopic studies of α_2M reveal a conformational change when α_2M reacts with an enzyme (31). This all demonstrates that the active site of an enzyme is not involved in the maintenance of binding to α_2M.

The physiological significance of α_2M lies most probably in a clearing function for proteinases from the circulation. Enzymes bound to other plasma inhibitors, dissociate slowly and are then irreversibly bound to α_2M. In vivo experiments showed that α_1-proteinase inhibitor is slowly transferred to α_2M (32). The α_2M-proteinase complexes are cleared very rapidly by the reticuloendothelial system. The half life is about 1o min in man (32).

PLASMA INHIBITORS

A list of the proteinase inhibitors that occur in blood is given in, Table 2. Their physiological function in controlling the proteolytic processes in the coagulation and fibrinolytic system, as well as in the kinine and hormone liberating and complement system have been reviewed (33-35). Thus antithrombin III (as a complex with heparin) and α_2M are the primary physiological inhibitors of thrombin, while α_2M and the fast reacting α_2-antiplasmin are the antagonists of the plasmin formed in plasma. It is striking that the thrombin-antithrombin III reaction is relatively slow in the absence of heparin which greatly accelerates the reaction (36). Heparin is released once the enzyme-inhibitor complex is formed and may further accelerate another reaction (37). The complete amino acid sequence of antithrombin III was reported (38), but almost nothing is known about the reactive site and the mechanism of enzyme-inhibitor association. The partial amino acid sequence of the α_1-proteinase inhibitor seems to indicate a sequence homology to antithrombin III.

The α_1-proteinase inhibitor (a_1PI, formerly α_1-antitrypsin) is the most studied plasma inhibitor. It is believed to be predominantly directed against leucocytic elastase, but inhibits as well trypsin and chymotrypsin. All enzymes may be inhibited at the same reactive site for which a Met-Ser peptide bond was suggested in the N-terminal part of the molecule (39). Interestingly oxidation of Met to the sulfone inactivates the inhibitory activity against leucocytic elastase (4o). It has been revealed that oxidants in cigarette smoke (perhaps together with myeloperoxidase from leucocytes) significantly lower the anti-elastolytic activity in serum (Jancff, personal communication). This increases the predisposition for emphysematic deseases.

Individuals homozygous in the genetic defiency variant carrying the ZZ allele in contrast to the normal MM allele are strongly predisposed to emphysema and to liver desease (41). Partial sequence studies revealed that a Glu residue in the M-protein is substituted by Lys in the Z-protein (42-44). This seems responsible for the depression of the plasma level of a_1PI up to 2o% of the normal concentration.

The recently discovered α_2-antiplasmin (45-47) reacts with plasmin extraordinarily fast. The strong lysine binding site on the heavy chain of plasmin seems to speed up the reaction by mediating a special interaction between α_2-antiplasmin and plasmin (48). In contrast α_1PI reacts with plasmin in a slow and progressive reaction (33). The exact mechanism of the interaction of enzymes and plasma inhibitors is not yet revealed. There is evidence that the mode of interaction may be similar to that elucidated for proteinases and low molecular weight inhibitors of animal or plant origin (see below), such as the basic bovine inhibitor (Kunitz), BPTI, or Trasylol®, which originates from mast cells (49). This view is supported by

studies on inter-α-trypsin inhibitor.

The acid labile inter-α-trypsin inhibitor of human plasma (MW 16o,ooo) Table 2, releases an acid stable proteinase inhibitor fragment (MW 14,ooo) from its C-terminus when treated with a number of proteinases, e.g. trypsin, plasmin, glandular kallikrein, and granulocytic elastase (5o). This inhibitor fragment consists of two tandem domains each of them homologous in its amino acid sequence to the low molecular weight bovine inhibitor (Kunitz), BPTI (51-54) Fig. 4 . This is an example of a two domain, double headed inhibitor (two independent active sites) evolved from gene elongation by duplication. The first domain has Met in its reactive site, however it does not inhibit any of the enzymes tested against it. The second domain contains Arg and inhibits trypsin. The fragment inhibitor is also liberated in vivo and is found in serum, rapidly excreted into the urine, where it has been identified as mingin (55).

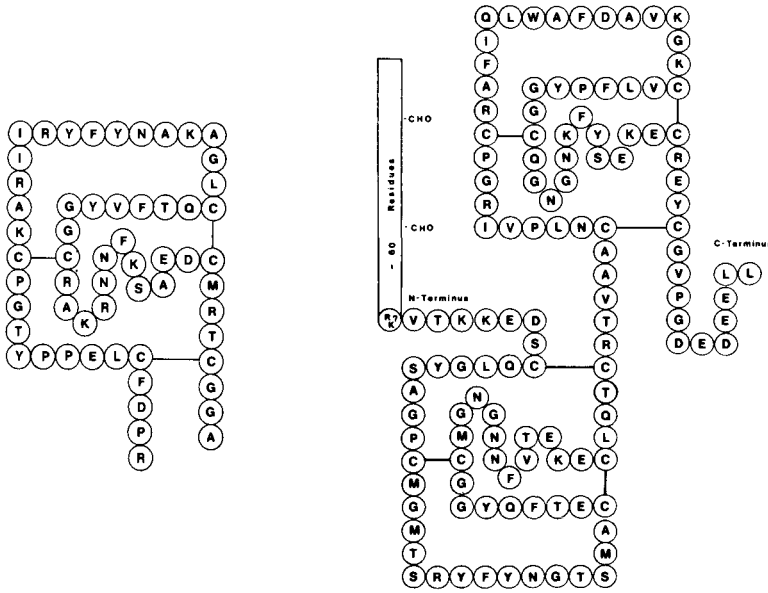


Fig. 4. Covalent structures of the two domain inter-α-trypsin inhibitor fragment and the bovine inhibitor (Kunitz), shown on left.

PROTEIN PROTEINASE INHIBITORS OF LOW MOLECULAR WEIGHT

About ten families of low molecular weight protein proteinase inhibitors are distinguishible (9-11) each consisting of several homologous inhibitors of serine proteinases, Table 3. All these are assumed to react according to a general mechanism. The overall mechanism of interaction which includes the so far established intermediates can be written:

$$E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons X \rightleftharpoons L^* \rightleftharpoons E + I^*$$

where E is the enzyme, I and I* are virgin and modified inhibitor, and L or L*

are loose, noncovalent complexes of E with I and I*, respectively. X is an intermediate in the E + I* reaction and C is the stable enzyme-inhibitor complex (9-11). The complex can either dissociate to give virgin inhibitor (P_1-P_1'intact) or modified inhibitor I (P_1,P_1' hydrolyzed). The equilibrium constant between modified and virgin inhibitor is near unity (9-11). The modified inhibitor can still form the complex, however in all cases studied with much lower rate. For this interaction the value k_{cat}/k_M is large with both k_{cat} and K_M many orders of magnitude lower than for normal substrates (9,56). On the surface of the substrate-like inhibitor is a specific peptide bond P_1-P_1' designated the reactive site which interacts with the active site of the target enzyme. The amino acid side chain of P_1 is inserted into the specificity pocket of the enzyme. The complex is stabilized by secondary forces, e.g. numerous van der Waal's interactions, hydrogen bonds, charge transfer interactions and as in the bovine inhibitor (Kunitz)-trypsin association by a salt bridge. The association energy is high with 1o-2o kcal/mole resulting in equilibrium constants for the association of $1o^7$ to $1o^{13}$ M^{-1}.

TABLE 3 Families of Protein Proteinase Inhibitors of Serine Proteinases with Low Molecular Weight

I.	Bovine pancreatic trypsin inhibitor (Kunitz) family
II.	Pancreatic secretory trypsin inhibitor (Kazal) family
III.	Streptomyces subtilisin inhibitor family
IV.	Soybean trypsin inhibitor (Kunitz) family
V.	Soybean proteinase inhibitor (Bowman-Birk) family
VI.	Potato I inhibitor family
VII.	Potato II inhibitor family
VIII.	Ascaris trypsin inhibitor family
IX.	Other families

The molecular nature of the complex is not yet fully clear. High resolution X-ray investigations on the trypsin complex of bovine inhibitor (Kunitz) (57) revealed that the reactive site peptide (P_1-P_1') is still intact. However, the carbonyl carbon of P_1 is no longer trigonal, but appreciably pyramidalized by the influence of the "oxanion hole", the NH's of Gly 193 and Ser 195 (58), of trypsin. This partial tetrahedral distortion was formerly interpreted as a true tetrahedral intermediate being formed. However, ^{13}C high resolution 36o MgHz NMR investigations on trypsin complexes with semisynthetic bovine inhibitor (Kunitz) (59) and soybean inhibitor (Kunitz) (6o, 61) highly enriched in the P_1 carbonyl carbon with ^{13}C indicated that a true tetrahedral transition state intermediate is obviously not present in the complex.

The reactive site of the inhibitor is located in a very rigid structure and the interaction with the enzyme is comparable to the classical picture of a key and lock interaction. A disulfide bond in most of the inhibitors links the reactive site peptide sequence into a loop and ensures that upon conversion of virgin to modified inhibitor the newly formed peptide chains cannot dissociate. The binding of an inhibitor is a matter of the proper surface geometry at the subside contact residues directed against the enzyme and is dominated by the type and space requirements of the reactive site amino acid P_1. In most cases, as a rough rule, the reactive site residue P_1 corresponds to the specifity of the target enzyme. Inhibitors with P_1 Lys or Arg inhibit enzymes with trypsin-like specifity, those with P_1 Tyr, Phe Trp, Leu and Met inhibit chymotrypsin-like enzymes, and those with P_1 Ala and Ser elastase-like proteinases. Semisynthetic substitution of P_1 Arg 63 in soybean trypsin inhibitor (Kunitz)and P_1 Lys in bovine inhibitor (Kunitz) by Trp

using an enzymatic replacement method (1o) lead to the conversion of a good trypsin inhibitor to a good chymotrypsin inhibitor (62, 63).

Using peptide synthetic methods it is possible to a certain extent to intentionally change the specifity of the bovine inhibitor (Kunitz) by substitution of the P_1 residue, Table 4, (64): Trp, Phe or Met in P_1 position lead to potent chymotrypsin inhibitors, Leu gives an excellent inhibitor of pancreatic and leucocytic elastase and chymotrypsin (with very little trypsin inhibition), while Val gives a strong inhibitor of leucocytic elastase (with weak inhibition of trypsin, chymotrypsin and pancreatic elastase). The anti-elastase activity is extremely weak (K_D = 1µM) in the native inhibitor but is newly introduced by substitution of the P_1 residue. Protein inhibitors are subject to change of their biological activity by natural or semisynthetic "mutation", quite different to most other proteins, where the reactive sites are strongly preserved during evolution and substitution leads to the loss of activity.

TABLE 4 Inhibitory Specificity of P_1 Substituted Bovine Inhibitor (Kunitz)

Reactive Site	Inhibited Proteinase			
Residue P_1	Trypsin	Chymotrypsin	Pancreas-	PMN-Elastase
Lys	+++	++	o	(+)
Arg	+++	++	o	o
Trp	++	+++	o	o
Phe	++	+++	o	o
Leu	+	+++	+++	+++
Met	+	+++	+	+
Val	+	+	+	+++
Ala	+	+	+	+
Gly	+++	++	o	o

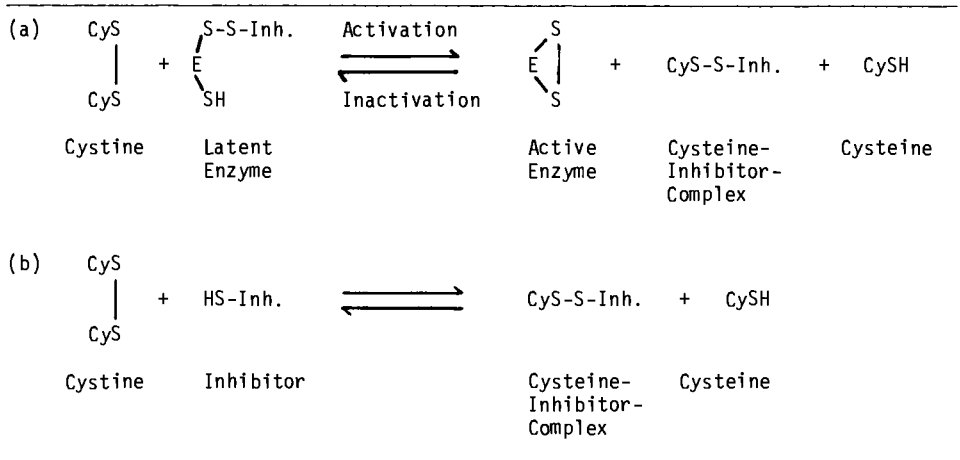
Inhibition: +++, strong; ++, medium; +, little; o, not detectable.

INHIBITORS REACTING BY DISULFIDE - INTERCHANGE

For a long time a controversy has existed whether latent enzymes such as latent mammalian collagenases exist as proenzymes activatable by active proteinases or exist as enzyme inhibitor complexes. Liberation of the active enzyme by zymogens, inactive enzymes, cystine, oxidised glutathione, insulin, or mercury compounds led us to the conclusion (65) that an inhibitor bound by a disulfide link is removed in a reversible disulfide interchange reaction from the latent enzyme, see scheme. The inhibitor (MW 24.ooo) could be separated and containes a single sulfhydryl group which is essential for activity. The inhibitor combines with the activating protein (active or inactive proteinases) such as trypsin to give an inactive trypsin-inhibitor complex. It can also be used to titrate active collagenase which then recombines in an disulfide interchange reaction to yield again the latent enzyme (65). The inhibition of the proteinase seems likely to be caused by reaction with a disulfide bridge in the vicinity of the enzymes reactive site leading to steric hindrance in substrate binding. This conclusion comes from the fact that 179, 2o3-di-S-carboxymethyl-trypsinogen is unable to activate latent collagenase (65). These rather recent experiments are supported by the findings of Steven and others (66,67),who explain the inhibition of a trypsin dependent neutral protease from malignant tissue by its inhibitor and its activation by mercury compounds

with the same mechanism. It remains at present unknown if the two inhibitors are identical or are the first members of a new mechanistic class of proteinase inhibitors.

SCHEME



PROTEINASE INHIBITOR AS DRUGS

Critical reviews on the use of proteinase inhibitors as potential drugs have recently appeared (68,69). Physiological and clinical studies with leupeptin and pepstatin have been reported (13). Many suggestions and expectations on their potential use have been made: e.g. leupeptin as anti-inflammatory agent, for treatment of burns, for acute pancreatitis and as an inhibitor of kinin formation; pepstatin for treatment of stomach peptic ulcer, muscular dystrophy, cartilage disorders such as osteoarthritis and therapy of hypertension. Both compounds have low toxicity, however, their clinical value as drugs has not yet been proven and pepstatin and leupeptin have been reported to exhibit teratogenic effects (70,71).

The inhibitor (Kunitz) with broad specificity from bovine lung, i.e. Trasylol^{R}, has found widespread application in medicine. Its usefulness is not without doubt, but it has been shown to reduce shock symptoms when administered systemically. The inhibition of kininogenases reduces the release of kinins (72) and rapid relief from pain in acute pancreatitis has been reported. The therapy of acute haemorrhage in gynecology is possible due to its strong inhibition of plasmin.

Inhibitors of the renin-angiotensin-system have recently been developed to powerful drugs in the therapy of hypertension (69). The renin-angiotensin system (73) participates in the maintenance and elevation of blood pressure. The kidney enzyme renin is released into the blood from the kidney in response to hyponatremia or low blood pressure. It is the rate limiting enzyme that cleaves the blood glycoprotein angiotensinogen (AO) between residue Leu 1o and Leu 11 to liberate the biologically inactive decapeptide angiotensin I (AI). Circulating AI is converted in kidney, lung and other tissues to the pressor octapeptide angiotensin II (AII) by the angiotensin converting enzyme (ACE), a carboxypeptidyldipeptidase (EC.3.4.15.1, kininase II), Fig. 5. The same enzyme (ACE) is responsible for the breakdown of the nonapeptide bradikinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg that is released from HMW- or LMW-kininogen by plasma or glandular kallikrein, respectively (74). Bradi-

kinin has a dilatory effect on arterial blood vessels and lowers the blood pressure. A number of natural pentapeptides acting as inhibitors of the converting enzyme (ACE) have been isolated from the venom of the Brazilian pit viper Bothrops jararca (75). The amino acid sequence of six components of the peptide mixture were determined (76-78), one component BPP_{5a} has the sequence: Pyroglu-Lys-Trp-Ala-Pro. These peptides block the degradation of bradikinin by ACE, prolonging the action of bradikinin (bradikinin potentiating peptides) and block the liberation of angiotensin II by C-terminal cleavage of the dipeptide His-Leu from angiotensin I. Thus, the ACE-inhibitor exhibits a twofold hypotensive effect in blocking the angiotensin liberation from the renin-angiotensin system and inhibiting the breakdown of bradikinin from the kallikrein-kinin-system. A synthetic dericative: D-3-mercapto-2-methylpropanyl-proline, with some main features of the structure of the bradikinin potentiating peptide was designed. It has a high inhibition constant of 1.7×10^{-9} M for ACE. This compound commercially available under the trade name captopril is an orally active antihypertensive drug, Fig. 6 (69).

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-R	AO
↓renin	
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	AI
↓ACE	
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	AII
↓angiotensinase A	
Arg-Val-Tyr-Ile-His-Pro-Phe	AIII

Fig. 5. Liberation of Angiotensin.

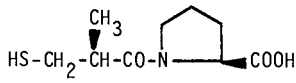


Fig. 6. Captopril

In the latter case the research on proteinase inhibitors has led to the development of a useful drug. The useful application of proteinase inhibitors suffers from various kinds of problems, e.g. that enzymes often perform necessary functions at one location, but being harmful to the body at another place, or that several proteinases act together in a concerted reaction and selective inhibition of just one enzyme remains ineffective. In conclusion proteinase inhibitors are a potentially useful class of drugs.

ACKNOWLEDGEMENT:

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Sialidases and Their Inhibitors

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ABSTRACT

The occurrence of sialidases is considered together with their substrate specificities. Sialidase inhibitors are divided up into four groups, natural high and low molecular weight compounds, synthetic compounds, and inorganic and non-specific reagents. The biological and pathophysiological functions of sialidases in relation to inhibitors, especially in mammalian systems, is discussed.

KEYWORDS

Sialidases; inhibitors; microorganisms; mammals; sialic acids; complex carbohydrates.

SIALIDASES AND THEIR INHIBITORS

The group of enzymes known as the sialidases (neuraminidases) is widespread in nature, being found in pathogenic and non-pathogenic bacteria, e.g. Clostridium perfringens, Vibrio cholerae and Arthrobacter ureafaciens, the myxoviruses, e.g. influenza viruses, mycoplasma, fungi, several protozoa, e.g. Trichomonas foetus, and is widely distributed in mammalian tissues (Drzeniek, 1972; Gottschalk and Drzeniek, 1972; Rosenberg and Schengrund, 1976). Characterization of sialidases in bacteria, where the enzyme is often secreted and can be induced, and in viruses, where the enzyme is invariably membrane-bound, is less complicated than the situation in mammalian tissues. Here the occurrence of lysosomal, Golgi and plasma membrane-bound enzymes, as well as soluble activity, is known (Corfield, Michalski and Schauer, 1981; Gottschalk and Drzeniek, 1972; Rosenberg and Schengrund, 1976). Studies on the bacterial and viral enzymes have detected a marked substrate specificity relating to the class of substrate and the type of linkage between the sialic acid and the neighbouring monosaccharide (Drzeniek, 1972, 1973). Mammalian sialidases also show such substrate specificity and this can be positively correlated with the glycoconjugate metabolism in that tissue. These properties relate to the probable function of the sialidases. Mention of the groups of substrates available to

sialidases is important, as inhibition is often related to the substrate employed. All glycoconjugates containing sialic acids in α-ketosidic linkage are potential sialidase substrates. Thus, oligosaccharides, such as found in urine, milk and colostrum (e.g. sialyllactose); polysaccharides (e.g. colominic acid); glycoproteins of plasma type (e.g. α_1-acid glycoprotein), mucus type (e.g. submandibular gland mucins) and membrane type (e.g. glycophorin A); gangliosides; and the linkage region of some proteoglycans, are included (Corfield, Michalski and Schauer, 1981; Gottschalk and Drzeniek, 1972; Rosenberg and Schengrund, 1976). In these groups the sialic acids occur in 2-3, 2-4, 2-6, 2-8 or 2-9 glycosidic linkages. Some of these substrates contain different glycosidic linkages in one molecule, thus exhibiting a potential for great specificity in sialidase action.

Several features of the sialic acid molecule in α-glycosidic linkage are important for sialidase activity. These are outlined in Fig. 1 and form a basis for sialic acid-derived sialidase inhibitors.

A number of sialic acids occurring in nature are known to be resistant or partially resistant to sialidase action and are also discussed as inhibitors below. These include 4-O-acetyl-N-acetyl (or -glycolyl)-neuraminic acids in gangliosides as well as in plasma and mucus glycoproteins derived from equine tissues (Schauer and others, 1980). These substrates are acted on only by virus and equine liver sialidases, but at greatly reduced rates relative to the saponified substrates (Sander, Veh and Schauer, 1979 a, 1979 b; Schauer and others, 1980). Metabolic studies with 4-O-methyl-N-acetylneuraminic acid transferred to fetuin showed deviations from the known behaviour of 4-O-acetylated sialic acids and are the first probes into an understanding of this type of resistance to sialidase cleavage (Beau and Schauer, 1980). O-Acetylation of sialic acids in the 7-9 carbon side chain hydroxyl groups results in about 50 % reduction as determined e.g. 9-mono-O-acetyl sialic acids (Schauer and Faillard, 1968). A further example is the branch or side chain sialic acid moiety of the gangliosides II^3Neu5Ac-GgOse$_3$-Cer and II^3Neu5Ac-GgOse$_4$Cer. These compounds are not substrates for many sialidases, and reduced rates of cleavage are found relative to other gangliosides with other sialidases,which may be enhanced in the presence of bile salts (Saito, Sugano and Nagai, 1979; Schauer and others, 1980). The reasons for this non-susceptibility are believed to be steric (Drzeniek, 1973; Schauer and others, 1980).

For the purpose of this survey the sialidase inhibitors have been divided up into four groups.

1. Naturally Occurring, High Molecular Weight Inhibitors

Several natural high molecular weight polyanionic polymers are potent inhibitors of viral sialidase activity. These include RNA, DNA, heparin and the potential substrate porcine submandibular gland mucin (Drzeniek, 1966). The effect is most likely non-specific, as synthetic polyanionic polymers also show this effect and reversal of the inhibition can be achieved using polycations, e.g. protamine (Drzeniek, 1972). The bacterial sialidases are less susceptible to this inhibition (Gottschalk and Drzeniek, 1972).

Another non-specific inhibitory compound, the D-glucose-, D-mannose-specific lectin Concanavalin A, has been found to inhibit influenza A virus sialidase, and a role for the carbohydrate moiety of the sialidase proposed (Zalan, Wilson and Freitag, 1975). Experiments with

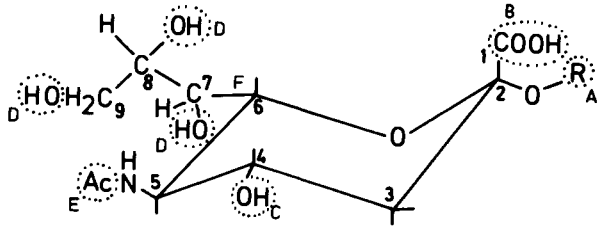


Fig. 1. The sialic acid (Neu5Ac) molecule and sialidase activity.

Moiety	Influence on Sialidase Activity	Literature
A Aglycone or linkage to next monosaccharide	Aglycone specificity related to hydrophobicity. Hydrolysis of 2-3, 2-6 and 2-8 linkages is enzyme specific and varies with the nature of the linked monosaccharide	Keilich, Ziegler and Brossmer, 1979. Drzeniek, 1972, 1973.
B Carboxyl function	Essential for activity. Esterification blocks cleavage by sialidase	Gottschalk and Drzeniek, 1972.
C Carbon 4 hydroxyl	O-acetylation results in abolition of or large reduction in cleavage. 4-O-methyl is less effective	Schauer and Faillard, 1968. Beau and Schauer, 1980.
D Carbons 7, 8 and 9 hydroxyl	O-acetylation results in reduction of cleavage rate, but no abolition of activity	Schauer and Faillard, 1968.
E N-acyl function	Rate of cleavage for N-acetyl > N-glycolyl in natural substrates. Larger and smaller synthetic groups lead to reduced activity	Corfield and others, 1980 b; Faillard, Ferreira do Amaral and Blohm, 1969; Brossmer and Nebelin, 1969.
F Carbons 7-9 side chain	Shortening to C_8 and C_7 may influence the rate of cleavage $C_9 > C_8 \gg C_7$	Veh and Schauer, 1978. Suttajit and Winzler, 1971.

Vibrio cholerae (Lamont and Isselbacher, 1977) and Clostridium perfringens sialidases (Table 1) indicate no influence on bacterial enzymes.

A sialidase inhibitor (neuraminin) produced by Streptomyces sp. No 289 (Lin, Oishi and Aida, 1975) shows more specificity than the previous inhibitors. Neuraminin is composed of 88 % carbohydrate as glucose and mannose, 12 % protein and has a molecular weight of 10^5. The inhibitor is active against viral but not bacterial sialidases (Lin, Oishi and Aida, 1977). With this inhibitor a further discrimination was found for influenza viruses which were not inhibited with sialyllactose or colo-

minic acid as substrates in contrast to fetuin and α_1-acid glycoprotein. The Newcastle disease virus sialidase was inhibited by neuraminin using all of these substrates. In all cases a non-competitive inhibition was found (Lin, Oishi and Aida, 1977).

TABLE 1 Binding of Clostridium perfringens Sialidase to Immobilized Inhibitors (and Substrates)
(Corfield, do Amaral Corfield and Schauer, unpublished)

Immobilized Inhibitor (or Substrate)	Inhibitory Activity	Binding
Concanavalin A (Lectin)	-	-
Wheat Germ Agglutinin	?	-
Neu5Ac2-6GalGlcNAc(Man)$_2$GlcNAc	(+)	-
α_1-Acid Glycoprotein (Substrate)	-	+
Fetuin (Substrate)	-	+
Ovomucoid	(+)	++
Porcine Submandibular Gland Mucin (Neu5Gl)	++	+++
Equine Submandibular Gland Mucin (Neu4,5Ac$_2$, Neu5Gl)	++	+++
Bovine Submandibular Gland Mucin, saponified (Neu5Ac, Neu5Gl)	+	+
Colominic Acid	+	-
2-Deoxy-2,3-dehydro-N-acetylneuraminic acid	+++	+
Neuraminic Acid-ß-methyl glycoside	-	+

- negative, (+) minimal, + -> +++ weak to strong

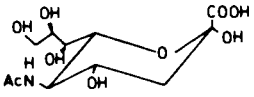
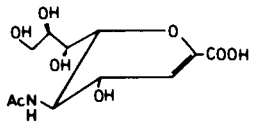
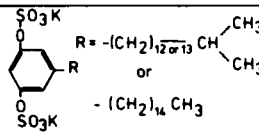
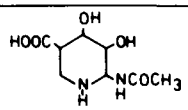
The interaction of ganglioside II^{3}Neu5Ac-GgOse$_4$Cer mentioned above has been studied with viral, bacterial and mammalian sialidases and a specific inhibition of the enzymes detected (Cestaro, Barenholz and Gatt, 1980; Sandhoff and Pallmann,1978; Schauer and others, 1980). This ganglioside forms micellar structures with a molecular weight greater than $2x10^5$. The inhibition has been analyzed as hydrophobic interactions which may influence the catalytic site of the enzyme, and was supported by the observation that the oligosaccharide II^{3}Neu5AcGgOse$_4$ was not an inhibitor (Corfield and others, 1980 a; Sandhoff and Pallmann, 1978).

A final class of macromolecular inhibitors may be the antibodies, but insufficient data and material are at present available for mammalian and bacterial sialidases. Viral sialidase antibodies have been widely used to monitor the occurrence and type of infections, e.g. influenza (Kilbourne, Christenson and Sande, 1968). Antibody studies with different sialidases and various substrates are required. The presence of endogenous mammalian sialidase inhibitors has not been demonstrated and also warrants closer investigation.

2. Naturally Occurring, Low Molecular Weight Inhibitors

A number of low molecular weight compounds act as sialidase inhibitors. These can be divided up into sialic acid derivatives and bacterial inhibitors with some similarity to the sialic acid molecule. The structures and inhibitory characteristics of these compounds are indicated in Table 2. The data should also be compared with the synthetic deri-

TABLE 2 Natural low molecular weight sialidase inhibitors

STRUCTURE	SIALIDASES viral	bacterial	mammalian	Lit
 N-ACETYL-D-NEURAMINIC ACID	Influenza A_2 (K_i, 5×10^{-3}M) Competitive	Vibrio cholerae (K_i, 5×10^{-3}M) Clostridium perfringens (-ve) Competitive	Human brain (30-40% inhibition at 10^{-2}M)	1,2, 3
 2-DEOXY-2,3-DEHYDRO-N-ACETYL-NEURAMINIC ACID	Influenza A_2 (K_i, 5.3×10^{-6}M) Influenza B (K_i, 9×10^{-5}M) Competitive	Vibrio cholerae (K_i, 3×10^{-5}M) Clostridium perfringens (+ve) Competitive	Human heart (K_i, 0.8×10^{-4}M) Human brain (K_i, 1.2×10^{-4}M) Rat heart (-ve) Human liver (+ve and -ve, two or more enzyme activities) Competitive	4,5, 6
 PANOSIALIN	Influenza (ID_{50}, 5×10^{-5}M) Newcastle disease (ID_{50}, 10^{-5}M) Non-competitive	NT	NT	7
 2-ACETAMIDO-3,4-DIHYDROXY-PIPERIDINE-5-CARBOXYLIC ACID (SIASTATIN B) SIASTATIN A (STRUCTURE NOT KNOWN)	Myxoviruses (-ve) (for A and B)	Vibrio cholerae (-ve) (for A and B) Clostridium perfringens (K_i, 1.7×10^{-5}M, B) (A > B) Streptomyces (K_i, 4.3×10^{-5}M, B) (A < B) Competitive	Chick chorioallantoic membrane (ID_{50}, 3.4 µg/ml, B) (A > B) Rat mammary gland (-ve) Rat brain (-ve) Rat liver (-ve) (for A and B) Competitive	8,9
$\alpha^{2,6}$Neu5Ac Lac SIALYL 2-6 LACTOSE	Newcastle disease (K_i, 10^{-3}M) Competitive	NT	NT	10

Literature 1. Walop, Boschman and Jacobs (1960); 2. Cassidy, Jourdian and Roseman (1965); 3. Veh and Schauer (1978); 4. Meindl and others (1974); 5. Parker, Veh and Schauer (1979); 6. Tallman and Brady (1973); 7. Aoyagi and others (1971); 8. Umezawa and others (1974); 9. Aoyagi and others (1975); 10. Drzeniek (1972).

+ve = inhibited, -ve = not inhibited, NT = not tested
The type of inhibition is indicated eg Competitive

vatives detailed in Table 3.

Sialic acid acts as a product inhibitor, but only at high concentrations (approx. 10^{-2}M), and in the case of Clostridium perfringens sialidase does not inhibit. The 2-deoxy-2,3-dehydro sialic acids are several orders of magnitude more potent (Tables 2 and 3) and deserve special attention, as 2-deoxy-2,3-dehydro-N-acetylneuraminic acid has been detected in normal saliva, urine and plasma in man (Haverkamp and others, 1976). Inhibition of mammalian sialidases has been observed for human heart and brain sialidases. However, the enzyme from rat heart (Tallman and Brady, 1973) and a glycoprotein-specific sialidase from horse liver (Sander, Veh and Schauer, 1979 b) are not inhibited. A further example of inhibition is presented in Fig. 2a for human liver sialidase (Michalski, Corfield and Schauer, unpublished), and is described below. In the case of virus sialidases, where 2-6 linkages are poorly cleaved, II^{6}Neu5Ac-Lac (and other 2-6-linked sialooligosaccharides) can function as an inhibitor (K_i = 10^{-3}M) (Drzeniek, 1972). Panosialin (Table 2) has not been investigated extensively and probably functions by a mixture of non-specific anionic and hydrophobic, detergent-like interactions, giving rise to non-competitive inhibition patterns (Aoyagi and others, 1971; Palese and Schulman, 1977). Siastatins A and B are competitive inhibitors with K_i values lower than the K_m for sialyllactose (Umezawa and others, 1974) and similar to values found for 2-deoxy-2,3-dehydro-sialic acids (Tables 2 and 3). No inhibitory action against viral sialidases was found. The specificity of inhibition in bacterial and vertebrate sialidases provides a tool for the investigation of the catalytic mechanism in different sialidases.

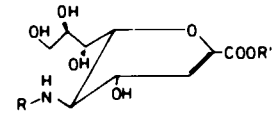
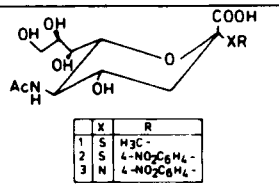
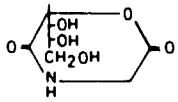
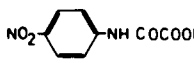
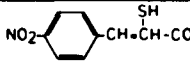
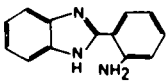
3. Synthetic Inhibitors

Investigations with 2-deoxy-2,3-dehydro-sialic acids containing different N-acyl moieties showed that inhibition could be markedly increased by substituting the N-acetyl residue by a trifluoroacetyl group (Meindl and others, 1974). Esterification of the carboxyl group at C_1 led to a complete loss of inhibitory activity (see also Fig. 1) and underlines the importance of this moiety for binding to the enzyme (Tuppy, 1974).

A series of 4-nitrophenyl glycosides of N-acetylneuraminic acid have been synthesized by Khorlin and others (1970), containing N and S atoms in place of oxygen. These compounds were potent inhibitors of Vibrio cholerae and influenza virus sialidases, while parainfluenza virus enzyme was poorly inhibited. The O-glycoside was active as a substrate. Another sialic acid analogue, 3-aza-2,3,4-trideoxy-4-oxo-D-arabino-octonic acid-δ-lactone proved to be a very strong inhibitor of bacterial and influenza virus sialidases. Low inhibitory activity was again found with the parainfluenza virus enzymes suggesting differences in the catalytic mechanism (Khorlin and others, 1970).

Very many organic compounds having no structural similarity to the sialic acids have been assayed for sialidase inhibition but only a few are useful inhibitors. Three examples of the most effective compounds from three classes of such compounds are given. The N-substituted oxamic acids have been extensively studied by Edmond and others (1966) and more recently by Brossmer, Keilich and Ziegler (1977). N-(4-nitrophenyl)-oxamic acid was the most inhibitory compound tested by Brossmer, Keilich and Ziegler (1977). Its immobilization through different moieties on the molecule onto Sepharose (Brossmer, Ziegler and Keilich, 1977; Cuatrecasas and Illiano, 1971) has provided a method for successful separation of two forms of Vibrio cholerae sialidase (Ziegler, Keilich and

TABLE 3 Synthetic sialidase inhibitors

STRUCTURE	SIALIDASES viral	bacterial	mammalian	Lit
 R = H_3CCO-, F_3CCO-, NCCO-, $HSCH_2CO$- and others; R' = H or H_3C- 2-DEOXY-2,3-DEHYDRO-N-ACYL-NEURAMINIC ACID	Influenza A Influenza B Newcastle disease (K_I, 10^{-6}M-10^{-4}M) R' = H- Competitive	Vibrio cholerae (K_I, 10^{-5}M-10^{-4}M) Clostridium perfringens (+ve) R' = H- R' = H_3C- (-ve) Competitive	Human brain (70-90% inhibition at 10^{-3}M) R = F_3CCO- R' = H- Competitive	1
 S and N-α-KETOSIDES OF N-ACETYL-NEURAMINIC ACID	% inhibition at 1.1×10^{-3}M Compound | 1 | 2 | 3 Influenza A | 37 | 0 | 18 Influenza B | 55 | 0 | 18 Parainfluenza type 2 | 76 | 59 | 41 Competitive	Vibrio cholerae (K_I, 10^{-3}M) 1 0.84 2 0.23 3 2.30 Competitive	NT	2
 3-AZA-2,3,4-TRIDEOXY-4-OXO-D-ARABINO-OCTONIC ACID δ LACTONE	% inhibition at 1.1×10^{-3}M Influenza A 100 Influenza B 100 Parainfluenza type 2 0 Competitive	Vibrio cholerae (K_I, 0.04×10^{-3}M) Competitive	NT	2
 N-(4-NITROPHENYL)-OXAMIC ACID	Influenza A 45% inhibition at 0.47×10^{-3}M Competitive	Vibrio cholerae (K_I, 0.58×10^{-3}M) Competitive	Human brain (40-70% inhibition at 10^{-2}M) Competitive	3,4
 β-(4-NITROPHENYL)-α-MERCAPTOACRYLIC ACID	NT	Clostridium perfringens (97% inhibition at 0.44×10^{-3}M) Competitive	NT	5
 1-(2-AMINOPHENYL)-BENZIMIDAZOLE	Influenza B (51% inhibition at 0.48×10^{-3}M) Competitive	Clostridium perfringens (62% inhibition at 0.48×10^{-3}M) Competitive	NT	5

Literature. 1. Meindl and others (1974). 2. Khorlin and others (1970). 3. Edmond and others (1966). 4. Brossmer, Keilich and Ziegler (1977). 5. Haskell and others (1970)

Brossmer, 1978) and removal of cytotoxic agents in commercial sialidase preparations (Den, Malinzak and Rosenberg, 1975). The exact nature of the interaction with sialidases is not fully understood and hydrophobic effects are discussed by Brossmer, Ziegler and Keilich (1977). In addition to the N-substituted oxamic acids simple aromatic compounds such as 4-nitroaniline, 2,4-nitrophenol and N-acetyl-4-nitroaniline showed K_i values in the range of $0.3\text{-}0.03 \times 10^{-3}$M for Vibrio cholerae sialidase (Brossmer, Keilich and Ziegler, 1977).

A number of ß-aryl-α-mercaptoacrylic acids have been found to inhibit Clostridium perfringens sialidase, the most effective being ß-(4-nitrophenyl)-α-mercaptoacrylic acid (Table 3) (Haskell and others, 1970). The same workers also found inhibition by 2-(3-aminophenyl)-benzimidazoles, but the action was not as high as for the ß-aryl-α-mercaptoacrylic acids. These classes of compounds were never assayed with mammalian sialidases (Haskell and others, 1970). Reports of inhibition by dihydroisoquinoline derivatives have appeared in the literature (Haskell and others, 1970; Tute, 1970), but this has been shown to be due to interference with the sialic acid assay method. Strong inhibition of rat heart sialidase was however found with 1-(4-methoxy-phenoxymethyl)-3,4-dehydroisoquinoline (60 % at 0.5×10^{-3}M) with a radioactive ganglioside substrate (Tallman and Brady, 1973).

None of these studies has produced a conclusive result and the proposals of hydrophobic interaction with sialidases, not involving the catalytic site (Brossmer, Ziegler and Keilich, 1977), probably apply to most cases. In keeping with the polyanionic macromolecular inhibitors discussed earlier, dextran sulphate is also an inhibitor, and the two azo-dyes, Congo red and Trypan red, probably function similarly, although their molecular weights are much smaller (Becht and Drzeniek, 1968; Drzeniek, 1966).

As a corollary to sections 1-3 the use of two inhibitors, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid and N-(4-nitrophenyl)-oxamic acid in a study of human liver lysosomal sialidase is presented in Fig. 2. This serves to illustrate the value of using different substrates in conjunction with various inhibitors and demonstrates the existence of at least two sialidase activities in this tissue.

4. Inorganic Ions, EDTA, Thiol Reagents and Detergents

Several bacterial (e.g. Vibrio cholerae) and viral (e.g. some influenza virus strains) sialidases show a requirement for divalent cations, usually Ca^{2+} (Corfield, Michalski and Schauer, 1981; Gottschalk and Drzeniek, 1972; Rosenberg and Schengrund, 1976). These enzymes are inhibited by EDTA, which chelates the divalent cations. The influence of monovalent ions varies considerably and a general effect with increasing ionic strength, also influenced by the pH value, occurs with polysialosubstrates (e.g. glycoproteins, polysialogangliosides) leading to repression of activity (see Rosenberg and Schengrund, 1976). Inhibition of mammalian sialidases varies with the source and location of enzyme in the cell and with the nature of the substrate (e.g. Leskawa and Rosenberg, 1980; Yeh and Carubelli, 1976). Thus, these factors must be considered when sialidase function in relation to ion inhibition or activation is studied.

Inhibition by Hg^{2+}, Cu^{2+} and Fe^{3+} from all groups of sialidases have been reported (see Corfield, Michalski and Schauer, 1981; Rosenberg and Schengrund, 1976) and may in part be correlated with the role of

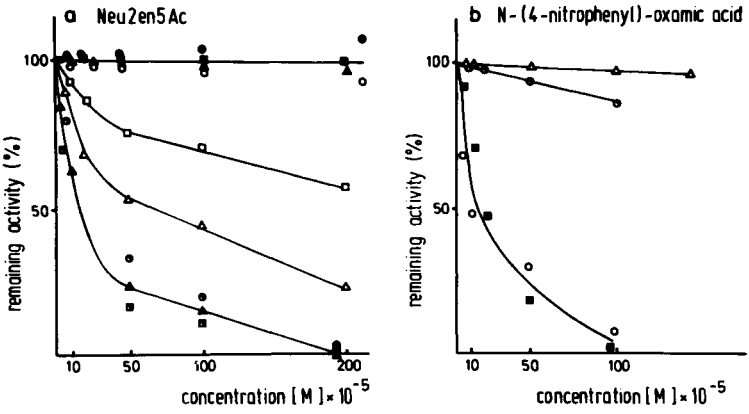


Fig. 2. Inhibition of human liver membrane-bound lysosomal sialidase by 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, a, and N-(4-nitrophenyl)-oxamic acid, b, with the following substrates: α_1-acid glycoprotein (●); fetuin (■); IV^{6}Neu5AcLcOse$_4$ (▲); Neu5Ac2-6GalGlcNAc(Man)$_2$GlcNAc (○); II^{3}Neu5AcLacCer (□); Ganglioside mixture (bovine brain) (△); II^{3}Neu5AcLac (◔); II^{6}Neu5AcLac (◧); Neu5Ac2-6Galß1-4GlcNAc (◭). (Michalski, Corfield and Schauer, unpublished).

SH groups in the enzymes. Inhibition by thiol compounds is essentially non-specific and has been observed with e.g. thioglycolate, glutathione and cysteine with bacterial and viral sialidases (Boschman and Jacobs, 1965; Rafelson, Schneir and Wilson, 1963).

Membrane-bound sialidases are subject to inhibition and sometimes activation by synthetic detergents such as Triton X-100 and bile salts (Gottschalk and Drzeniek, 1972; Rosenberg and Schengrund, 1976). These detergents also influence the glycolipid substrates and non-specific hydrophobic interactions play a major role. Investigations with phospholipids and ganglioside substrates in model membranes have illustrated that a membrane structure can influence the optimal activity of bacterial sialidases for these substrates (Cestaro, Barenholz and Gatt, 1980).

Biological Functions

Ascribing a biological function to sialidase inhibitors is as yet premature. In bacterial systems the production of sialidases may be related to the pathogenicity of the strain (Müller, 1974), an example being clostridial infection in gas gangrene with greatly increased sialidase activity in serum (Schauer, Jancik and Wember, 1979). In the oral cavity plaque formation and dental caries may be influenced by desialylation of salivary glycoconjugates (Perlitsh and Glickman, 1966), whereby a bacterial sialidase may play a role. The discovery of the inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid in normal human saliva, at

concentrations in some cases close to the K_i for sialidases (Haverkamp and others, 1976), presents a link in this direction, which is currently under investigation. The inhibition by mucus glycoproteins reflects the function of such molecules in saliva and epithelia in preventing colonization of the epithelia by microorganisms.

The antigenicity of viral sialidases has often been monitored after infection by virus-specific antibodies, and extension of this approach to general sialidase characterization still lags due to technical problems. Other viral sialidase inhibitors have been found to lack antiviral activity (Palese and Schulman, 1977) and may be metabolized before they can become effective. Some synthetic analogues of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid inhibit virus replication in cell culture, but no protective influence against influenza or parainfluenza virus infection in the intact host could be shown (Palese and Schulman, 1977).

In mammalian systems several examples of biological function for inhibitors await clarification. The sialic acids are widely distributed and biologically important molecules, their function in masking antigenic or receptor sites has been clearly shown. The removal of asialoglycoproteins from the circulation has been demonstrated (Ashwell and Morell, 1974), and a sialidase in serum detected (Schauer and others, 1976), but evidence for an endogenous sialidase inhibitor functioning in regulation is still missing. The phenomenon of erythrocyte ageing *in vivo* in which loss of sialic acid appears to be involved (Schauer, 1979) awaits conclusive demonstration of the mechanism with or without sialidase involvement, and the role of an endogenous inhibitor may again be considered. Sialidase may become a 'toxic' enzyme, if in excess, and in the case of gas gangrene cited above may function to indiscriminately 'demask' sites and receptors in host cells and fluids, thus disturbing the normal state. Future clinical use of specific sialidase inhibitors in such bacterial or viral infections can be envisaged.

An area of rapid development at present is that of the sialidoses. In these diseases sialidases may be deleted or malfunctional (Strecker and Montreuil, 1979), the opposite case to the excess enzyme found in gas gangrene. A recent hypothesis involves an endogenous factor influencing sialidase activity (Hoogeveen and others, 1980). This field may bring about the first insight into an intact mammalian system involving sialidases.

Further progress will depend on the characterization of sialidases, and this can be implemented using the inhibitors described here. Many inhibitors have been immobilized for use in enzyme purification (Table 1). The combination of suitable substrates with inhibition studies will be a powerful tool in understanding the mechanism of action of the sialidases, which is considered to be a prerequisite for the better understanding of the role of these enzymes in health and disease.

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Inhibitors of Cyclic Nucleotide Phosphodiesterases

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ABSTRACT

The interest in drugs which inhibit 3',5'-nucleotide phosphodiesterases is based on the mediatory role attributed to cyclic nucleotides in the intracellular effects of many hormones and neuromediators. Recently, several molecular forms of phosphodiesterase with different substrate specificities and sensitivities to Ca^{2+}-dependent activation by calmodulin were separated from many mammalian tissues. A new generation of inhibitors acting selectively on the various enzyme forms or preventing their activation by calmodulin was also found. These findings provide a basis for the search of more potent and specific inhibitors which would be useful tools in elucidating the respective role of the different phosphodiesterases in the regulation of cyclic nucleotide levels. The development of such compounds may also provide new therapeutical agents, since physio-pathological alterations of the metabolism of cyclic nucleotides have been reported.

KEYWORDS

Cyclic nucleotide phosphodiesterases ; cyclic AMP ; cyclic GMP ; calmodulin ; phosphodiesterase inhibitors ; calmodulin inhibitors.

INTRODUCTION

Cyclic nucleotide phosphodiesterases are the enzymes responsible for the intracellular degradation of 3',5'-nucleotides. A large number of chemical compounds with no obvious structural relationship have been reported to inhibit the breakdown of cyclic AMP and/or other cyclic nucleotides *in vitro*. These compounds, which include methylxanthines, isoquinolines, flavonoids, pyrrolidones, imidazolidinones, pyrazolopyridines, etc..., have been comprehensively reviewed by Amer and Kreighbaum (1975) and Chasin and Harris (1976). They display a wide spectrum of pharmacological actions, but the participation of cyclic nucleotides in these pharmacological actions is generally not clearly established. The main reason for this is that many if not all phosphodiesterase inhibitors have also other effects on various cell mechanisms (Stoclet and Lugnier, 1978 ; Stoclet, 1979, 1980a). Theophylline, for instance, which was the first discovered of the phosphodiesterase inhibitors (Sutherland and Rall, 1958), is approximately 100 times more potent as an adenosine antagonist than as a phosphodiesterase inhibitor (see

review by Stoclet, 1980b).

It is well established that, at least in isolated organs, suitable concentrations of phosphodiesterase inhibitors can potentiate some effects of hormones and neuromediators that stimulate adenylyl cyclase, and can reproduce the effects of exogenously added cyclic AMP (Sutherland, Robison and Butcher, 1968). These drugs may therefore provide a means to bring back to normal level a pathologically deficient cyclic AMP system. Such a deficience in the mediatory role of cyclic AMP has been implicated in various pathological states (for reviews see Amer, 1975 ; Weiss and Hait, 1977). However, the potential therapeutic possibilities afforded by inhibiting intracellular phosphodiesterases raise a number of question such as the following. Does the inhibitor act on the hydrolysis of one or several cyclic nucleotides ? Are its effects identical in all tissues ? Are its pharmacological and therapeutic properties mediated by one or several cyclic nucleotides ? It is not possible to examine here these questions in the case of each of the many drugs that have been reported to inhibit a 3',5-nucleotide phosphodiesterase activity. The purpose of this review is rather to discuss some recent findings relevant to the problems raised by the development of phosphodiesterase inhibitors as therapeutical agents. After an overview of our present knowledge on the phosphodiesterases, the principal classes of inhibitors will be presented. Some of the pharmacological properties of these drugs and therapeutical perpectives will be discussed at last.

CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

Different molecular forms of phosphodiesterase have been physically separated from pratically all mammalian tissues and characterized by their substrate specificity, their kinetic properties and their sensitivity to activators and inhibitors. These enzyme forms were reviewed by Wells and Hardman (1977), who pointed out the difficulties to summarize the results without oversimplification. This difficulty is probably partially due to the extreme diversity of methods employed and to the heterogeneity of the cells contained in the studied tissues. However it probably results also from the complexity of the enzyme system itself, since several enzyme forms could be separated from the media layer of large arteries, which contains only one cell type (Wells and co-workers, 1975 ; Ilien and co-workers, 1978). In addition, all studied tissues contain various proportions of an endogenous activator called calmodulin. It is a small thermostable protein with a high affinity for Ca^{2+}, which is emerging as an intracellular receptor for Ca^{2+} (Cheung, 1980). In presence of "physiological" (10^{-6} M) Ca^{2+} concentrations, it is able to activate many enzyme systems and thereley to regulate many cell functions.

Schematically, there are phosphodiesterase forms which can be activated by calmodulin and others which can not. One of the major forms found in most tissues is sensitive to calmodulin (Klee, Crouch and Krinks, 1979). It has only one active site hydrolyzing both cyclic AMP and cyclic GMP (Liang and co-workers, 1980), but it has a greater Vmax and a higher Km for cyclic AMP than for cyclic GMP (for this reason, it has sometimes been described as a "high Km" phosphodiesterase). Another form found in many tissues is sometimes describeb as the "low Km cyclic AMP" or "negatively cooperative" phosphodiesterase. It is characterized by relative selectivity towards cyclic AMP as substrate, lack of sensitivity to calmodulin, and non linear kinetics. Other forms have been separated from various tissues. For instance in our laboratory we also separated from bovine aorta mediacytes a phosphodiesterase which is activated by calmodulin and hydrolyzes identically cyclic AMP and cyclic GMP and a phosphodiesterase which is not sensitive to calmodulin and uses cyclic GMP as prefered substrate (Ilien and others, 1978).

We do not know whether the properties of the phosphodiesterases within cells are identical to those of the molecular forms that have been isolated. On one hand,

separation from endogenous factors may modify these properties. For instance, Davis and Daly (1980) recently reported that gangliosides increase rat cerebral cortical cyclic nucleotide phosphodiesterase activity and that the Ca^{2+}-dependent activity was more sensitive to gangliosides than the Ca^{2+}-independent activity. On the other hand the properties of the isolated enzyme forms may be artifactually modified during the extraction procedure (by endogenous proteases, for instance). In addition it should be mentioned that practically nothing is known on the hydrolysis of naturally occuring 3',5'-nucleotides other than cyclic AMP and cyclic GMP. Recently Kuo and co-workers (1978, 1979) reported that cyclic CMP phosphodiesterase is different from cyclic AMP and cyclic GMP phosphodiesterases and is not inhibited by the same drugs.

For the above mentioned reasons, the respective participation of the different phosphodiesterases to the intracellular regulation of cyclic AMP and cyclic GMP levels remains to be elucidated. Recently Barber and Butcher (1980) proposed a new method to estimate the turnover of cyclic AMP in cultured fibroblasts. If this method can be used in other cells, it may help to understand the role of the phosphodiesterases. At the present time it is only possible to speculate that the pathways of intracellular breakdown of each of the two cyclic nucleotides follow two different ways (Stoclet, 1979) : one is not sensitive to calmodulin and is relatively specific for its substrate ; the other can be activated by calmodulin and is less specific for the substrates. Release of activator Ca^{2+} in stimulated cells seems to trigger a complex regulation of cyclic nucleotide levels through the activation by calmodulin of both synthesis (by adenylate and guanylate cyclases) and degradation of cyclic AMP and/or cyclic GMP (see reviews by Cheung, 1980, and Demaille, 1980).

It can be concluded that there are two ways for a drug to inhibit cyclic AMP and/or cyclic GMP breakdown in cells : one is to inhibit directly one of the active sites hydrolyzing cyclic nucleotides, the other is to inhibit their activation by calmodulin (Weiss and Levin, 1978). These two mechanisms will be discussed below. It is important to point out that the different molecular forms of phosphodiesterase seem to be present in various proportions in the different tissues (Wells and Hardman, 1977 ; Weiss and Levin, 1978) and to vary in pathological states (see below). Thus, their selective inhibition might cause selective effects in tissues which contain a high proportion of the corresponding form. Furhtermore they might produce different effects on the level of the two cyclic nucleotides, depending on the degree of activation by Ca^{2+} of the calmodulin-sensitive form(s).

COMPETITIVE PHOSPHODIESTERASE INHIBITORS

As mentioned above, a large number of compounds inhibit the hydrolysis of cyclic AMP and/or cyclic GMP *in vitro* (Amer and Kreighbaum, 1975). However many of these compounds have not been assayed on purified enzyme forms and are only active at high concentrations which are not likely reached in cells in conditions producing pharmacological effects. Table 1 contains a selective list of the most active or the most widely used competitive inhibitors, some of which preferentially inhibit cyclic AMP or cyclic GMP hydrolysis. It should be mentioned, however, that the effective concentration ranges indicated in Table 1 correspond to those found in the indicated references, but may vary according to the tissue. For instance compound Ro 20-1724 is approximately 100 times more potent to inhibit cyclic AMP phosphodiesterase from erythrocytes (Sheppard and Wiggan, 1971) than cyclic AMP phosphodiesterase from brain (Schwabe and others, 1976). In this respect, it would be interesting to know if cilostamide is as potent to inhibit cyclic AMP phosphodiesterase in other cells than in blood platelets.

In our laboratory, we confirmed the relative selectivity of compounds such as papaverine and ZK 62711 on cyclic AMP phosphodiesterase and M + B 22948 on cyclic GMP phosphodiesterase, using purified enzyme forms from bovine aorta and lung. However we found that these compounds inhibited identically the hydrolysis of the two cyclic nucleotides by calmodulin-sensitive phosphodiesterase forms which also hydrolyzed both cyclic AMP and cyclic GMP at the same active site (Ruckstuhl and others, Stierle and others, in preparation). These findings support the view that selective phosphodiesterase inhibitors should modify differentially the level of intracellular cyclic nucleotides according to the free Ca^{2+} concentration in cells. In agreement with this hypothesis, we previously reported that the addition of increasing concentrations of external Ca^{2+} can overcome the elevation of cyclic AMP produced by papaverine in K^{+}-depolarized rat aorta strips (Demesy-Waeldele and Stoclet, 1977).

TABLE 1 Effective concentration range of selected cyclic nucleotide phosphodiesterase inhibitors

Substances	Effective concentration range (M) cyclic AMP	cyclic GMP	References
Purines :			
theophylline	10^{-6}	10^{-4}	Sutherland and Rall (1958)
MIX (a)	10^{-5}	10^{-6}	Wells and others (1975)
M + B 22948 (b)	10^{-4}	10^{-6}	Bergstrand and others (1977)
Isoquinoline			
papaverine	10^{-6}	10^{-5}	Lugnier and Stoclet (1974)
Dihydroquinoline			
cilostamide (c)	10^{-9}	10^{-6}	Hidaka and others (1979)
Flavonoid			
amentoflavone	10^{-7}	10^{-7}	Ruckstuhl and others (1979)
Imidazolidinone			
Ro 20-1724 (d)	10^{-5}	10^{-4}	Weinryb and others (1972)
Pyrrolidone			
ZK 62711 (e)	10^{-6}	10^{-4}	Schwabe and others (1976)
Pyrazolopyridine			
SQ 20009 (f)	10^{-6}	10^{-5}	Chasin and Harris (1976)

(a) 1-methyl-3-isobutylxanthine
(b) 2-0-propoxyphenyl-8-azapurin-6-one
(c) N-cyclohexyl-N-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy)butyramide
(d) 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone
(e) 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone
(f) etazolate hydrochloride ; 1-ethyl-4-(isopropylidenehydrazine)-1H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, hydrochloride.

As shown in Fig. 1, there is no obvious structural analogy between the various various competitive inhibitors and cyclic AMP or cyclic GMP. The figure represents one of the possible conformations of cyclic nucleotides as determined in cristallographic studies using X-ray diffraction (Watenpaugh et al., 1968 ; Drugan and Sparagana, 1976). In solution, the *anti* conformation is predominant in the case of cyclic AMP and there is a slight preference for the *syn* conformation of cyclic GMP, as shown by Magnetic Nuclear Resonance (Yathindra and

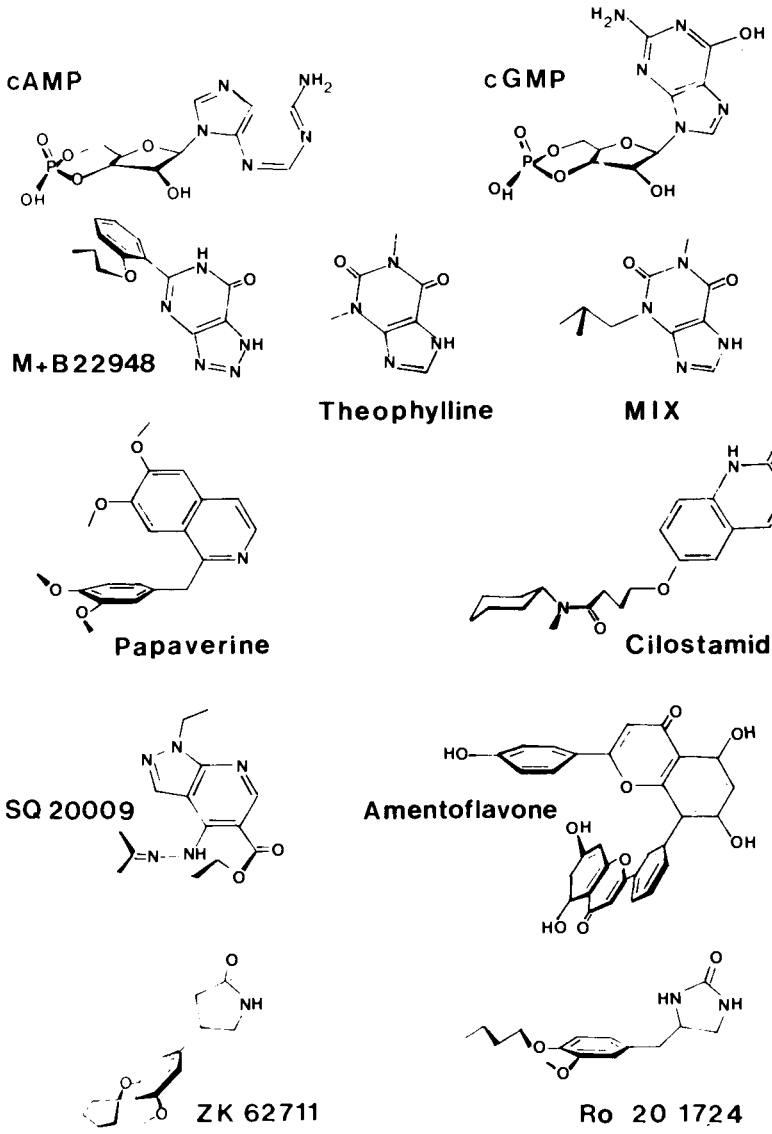

Fig. 1 Tentative representation of configuration of selected cyclic nucleotide phosphodiesterase inhibitors (same abreviations as in Table 1) as compared to one configuration of cyclic nucleotides.

Sundaraligan, 1976). The purine basis is in one plane and the rest of the molecule in another plane. No such studies have been performed on inhibitors. The tentative configurations of inhibitors represented in Fig. 1 rest on the hypothesis that, if they are competitive as suggested by enzymatic studies, they must take the same configuration as cyclic nucleotides to accede to the active site of the enzyme. Obviously, the xanthine nucleus may compete with the purine moiety of cyclic nucleotides and, interestingly, with other purine compounds such as adenosine. This is also probably the case for other phosphodiesterase inhibitors, the pharmacological properties of which may be due to interactions with adenosine and/or non cyclic purine nucleotides (see below).

CALMODULIN INHIBITORS

Weiss and co-workers (1974) reported that trifluoperazine and other phenothiazine antipsychotic agents selectively inhibit a phosphodiesterase form wich is Ca^{2+}-dependent and which is predominant in most areas of the brain. It has subsequently been shown that calmodulin is the activator protein responsible for the Ca^{2+}-dependence of this phosphodiesterase form and that trifluoperazine and related drugs act by binding to calmodulin rather than to the enzyme or Ca^{2+} (Levin and Weiss, 1977 ; Weiss and Levin, 1978). These drugs are therefore calmodulin rather than phosphodiesterase inhibitors, and they inhibit the biological properties of calmodulin such as activation of myosin light chain kinase and of other enzymes activities (Hidaka and co-workers, 1979b, 1980). As shown in Table 2, they include various chemical structures. They represent interesting tools to analyze the role of calmodulin, but it is doubtful that their interaction with calmodulin plays a part in their pharmacological and therapeutic properties, since most of them interact with catecholamine and other receptors at a much lower concentration than the concentration effective on calmodulin (Weiss and Levin, 1978 ; Stoclet and colleagues, in press).

TABLE 2 Calmodulin inhibitors and their effective concentration ranges (in M) on calmodulin and cyclic nucleotide phosphodiesterase activities (calmodulin sensitive form)

Substance	Calmodulin	Phosphodiesterase	Reference
Phenothiazine trifluoporazine	10^{-6}	10^{-4}	Weiss and Levin (1978)
Thioxanthene chlorprothixene	10^{-5}	10^{-3}	" "
Butyrophenone benperidol	10^{-5}	10^{-4}	" "
Diphenylbutylamine pimozide	10^{-6}	10^{-4}	" "
Benzodiazepine amitriptyline	10^{-4}	10^{-3}	" "
Dibenzazepine desipramine	10^{-4}	10^{-4}	" "
Imidazole	10^{-4}		Klee and others (1979)
Vinblastine	10^{-5}	10^{-4}	Wanatabe and others (1979)

PHARMACOLOGICAL PROPERTIES OF PHOSPHODIESTERASE INHIBITORS

The experimental design generally used to correlate a pharmacological effect with phosphodiesterase inhibition is based on the criteria initially proposed by Sutherland, Robison and Butcher (1968) in the case of adenylyl cyclase simulating agents. Before a phosphodiesterase inhibitor is presumed to act through a change in the intra-cellular concentration of a cyclic nucleotide :

1. It should inhibit the activity of PDE of the tissue(s) on which it exerts the studied effect(s) and should be able to do so at a concentration consistent with the hypothesis (unfortunately, inhibition of phosphodiesterase could be demonstrated *in vitro* only, except in the case of MIX using the method recently proposed by Barber and Butcher, 1980).

2. It should change the cyclic nucleotide content of intact cells in a dose dependent fashion and a time course consistent with a triggering action for the response (however total tissue content of cyclic nucleotides may be misleading because of the heterogeneity and the intracellular compartimentalization of most tissues).

3. It should potentiate the effects of agents that stimulate the corresponding cyclase. So far, adenylyl cyclase but not guanylyl cyclase is stimulated by hormones or neuromediators (it should be pointed out that many conflicting results have been reported on this point).

4. An exogenously administed cyclic nucleotide should mimick the pharmacological effect(s) (but the available cyclic nucleotide analogues which can be used also possess non specific effects).

The difficulties met to fulfill the criteria have been discussed elsewhere (Stoclet, 1978). The pharmacological properties associated with phosphodiesterase inhibition are cardiac stimulation (Korth, 1978), smooth muscle relaxation (Kramer and Wells, 1979 ; Kukovetz and co-workers, 1979) inhibition of platelet aggregation (Hidaka and co-workers, 1979) and inhibition of histamine and other mediators of anaphylaxis (Kaliner and Austen, 1974). Other properties such as modulation of pre-synaptic neuromediator release (Langer, 1977) or gastric secretion (Puurunen, Lücke and Schwabe, 1978) might also result from phosphodiesterase inhibition.

I should be stressed that fulfillment of the above discussed criteria does not rule out the participation of additional mechanisms. Theophyllin and MIX are potent adenosine antagonists (Schultz and Daly, 1973). Other phosphodiesterase inhibitors such as papaverine, SQ 20,009 and Ro 20-1724 inhibit uptake of adenosine (Mah and Daly, 1976). Much of the effects of these compounds may result from their interaction with the adenosine system, which influences adenosine sensitive adenylate cyclases in a complex fashion (Braun and Levitzki, 1979). These mechanisms may play a part in vascular and tracheobronchial smooth muscle relaxation (Stoclet and Lugnier, 1978 ; Fredholm, Brodin and Strandberg, 1979 ; Stoclet, 1980), in effects on brain (Schultz, 1974) and in platelet aggregation (Jakobs and co-workers, 1979), for instance. Other mechanisms such as interaction with prostaglandins (Levy, 1973 ; Horrobin and others, 1977), direct interactions with Ca^{2+} release (Martinez and Mc Neill, 1977) may also play a part in pharmacological effects of isoquinolines and methylxanthines.

THERAPEUTIC PERSPECTIVES AND CONCLUSION

With respect to potential therapeutic applications of cyclic nucleotide phosphodiesterase inhibitors, the principal question is to know whether these drugs might be used to bring back to normal pathologically altered cyclic nucleotide

levels. Reduced levels of cyclic AMP have been found in malignant or transformed cells (Millis and co-workers, 1972 ; Shepppard, 1972) and are in general associated with rapid proliferation (Otten and co-workers, 1972), whereas the concentration of cyclic GMP is increased in rapidly proliferating cells (Goldberg and co-workers, 1973). Low levels of cyclic AMP and/or reduced elevation of cyclic AMP content in response to hormonal stimulation have also been found in various pathological tissues, such as skin from patients suffering from psoriasis (Vorhees and others, 1973, 1975 ; Iizuka and others, 1978), adipocytes from obese mice (Kapiucki and Adams, 1974 ; Lovell-Smith and Sneyd, 1974), atherosclerotic lesions (Shimamato, 1975 ; Lundholm and others, 1980), pulmonary tissue from asthmatic patients (Krzanowski and others, 1979), atria from hypothyroid rats (Brodde, Schümann and Wagner, 1980), heart lesions in congestive heart failure (Gold and others, 1970), and arteries or platelets from hypertensive rats or patients (Amer and others, 1974 ; Hamet and others, 1978), for example. In most cases, however, the cause-effect relationships are not established, and the observed alterations in cyclic nucleotide level may be a consequence of the disease rather than an etiological fact. For instance the role of cyclic AMP in the growth of malignant cells is controversial, since only certain tissues *in vivo* have lower levels of cyclic AMP than their normal counterparts (Goldberg and others, 1975 ; Stevens and others, 1976).

Whatever be the cause of the above mentioned alterations in cyclic nucleotide metabolism (alterations in cyclase and/or phosphodiesterase activity have both been reported) the use of a specific phosphodiesterase inhibitor to bring back the cyclic nucleotide to normal level may provide a means to investigate the consequences of the lowered cyclic nucleotide level and its possible relevance to the pathological phenomenon. In a study on the various forms of phosphodiesterase from normal and leukemic lymphocytes, Hait and Weiss (1979) recently suggested the possibility of developing chemotherapeutic agents that act by selectively altering the metabolism of cyclic nucleotides in malignant tissues. These authors observed that dipyridamole inhibited phosphodiesterase from leukemic lymphocytes at a concentration having little effect on phosphodiesterase from normal lymphocytes.

The possibility of using phosphodiesterase inhibitors as therapeutical agents still raises a number of questions. The different phosphodiesterase forms that have been isolated from tissues can be selectively inhibited by newly developed compounds (Kramer and Wells, 1979) and they are distributed in various proportions in the different tissues. This provides a molecular basis to selective effects on different normal or pathological tissues. Pharmacokinetic factors may also which are not discussed here may also play a role in the effects on different tissues. However we do not know whether the phosphodiesterases in cells are identical to the forms which have been separated and purified, and what is their respective role. Furthermore the possible consequences of inhibiting phosphodiesterases in cells are to a large extent unknown. For instance phosphodiesterase activity increases during ontogenic development (Stancheva, Petrov and Uzunov, 1979) and during maturation (Lugnier and Stoclet, 1979). The synthesis of one phosphodiesterase form is also increased by noradrenaline and other cyclic AMP elevating agents (Uzunov, Shein and Weiss, 1973). Recently, Thompson and others (1980) reported that addition of MIX to human lymphocytes in culture caused an increase in h high affinity form of cyclic AMP phosphodiesterase, which reached a maximum after 48 h of contact.

Such an increase in phosphodiesterase activity might occur during treatment by phosphodiesterase inhibitors and it is not possible at the present time to predict its possible consequences. One may speculate, however, that these consequences might be important since phosphodiesterase activity undergoes physiological variations in cells.

Despite the many important problems which remain to solve before the possible therapeutical interest of inhibiting cyclic nucleotide phosphodiesterase can be evaluated, it seems possible to conclude that the recent development of compound which potently and selectively inhibit the different phosphodiesterase forms that have been isolated provide means to investigate these problems. Such investigations may open the way to therapeutical applications in diseases in which cyclic nucleotide metabolism is altered.

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The Systematics of Bioactive Peptide Structure-Activity Relationships

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ABSTRACT

Structural modifications of six bioactive peptides are discussed in respect of their effect on potency, selectivity, protracted action and in the generation of competitive antagonism.

KEYWORDS

Peptides; structure-activity relationships; potency; antagonism; selective action; protracted action.

INTRODUCTION

It would be presumptive of any author to claim that systematics have been established in the field of structure-activity (S-A) relationships of bioactive peptides. Attempts at systematics have, nevertheless, been the basis for significant achievements, and provide an instructive basis for new investigations. Let us first consider the two main ingredients of my subject, i.e. 'structure' and 'activity', so that the selections I must necessarily make can be seen in perspective.

Structural modifications of linear peptides investigated by peptide chemists have been of three main types:-

1. Changes in chain length by adding or subtracting amino-acid residues. These may arise (a) by extensions at the N- or C-terminus, when the parent sequence is maintained intact, (b) by deletions at the N- or C-terminus, which have usually served to define a 'minimal fragment' for biological activity, or (c) by insertions or deletions within the peptide chain, which have served to ascertain the effect of 'frame shifts' of sequences within the whole sequence.
2. Modification of side chains by replacement of individual amino-acid residues by other natural amino-acid residues or by unnatural residues.
3. Modification of the peptide backbone. The following types of modification are representative of those pursued (see Fig 1).

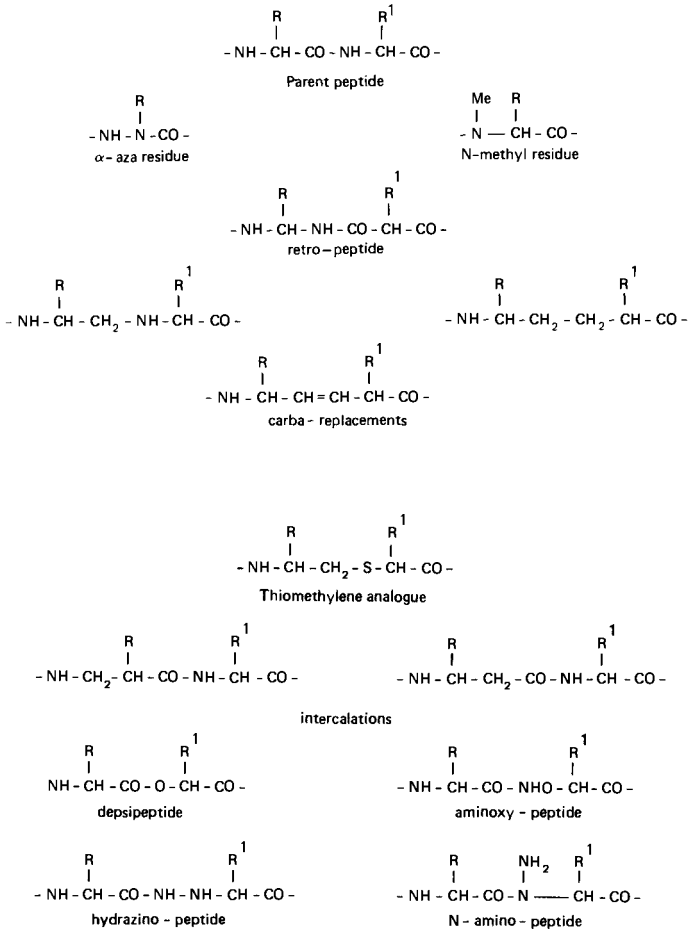


Fig. 1. Some modifications of the backbone

(a) Configurational change at α-carbon atoms by replacement of L- by D-residues, or by α-aza amino-acids (α-CH replaced by N).
(b) N-Methylation of the peptide bond (CO-NH replaced by CO-NMe).
(c) Reversal of the peptide bond (CO-NH replaced by NH-CO), i.e. retro-peptides.
(d) Carba replacements of peptide or, when Cys is present, disulphide bonds (CO-NH replaced by CH_2-CO, CH_2-NH, CH_2-CH_2, or CH=CH; S-S replaced by CH_2-S or CH_2-CH_2).
(e) Thiomethylene analogues (CO-NH replaced by CH_2-S), sometimes called 'peptide gap' inhibitors.
(f) Intercalation of CH_2 (NH-CHR-CO replaced by NH-CH_2-CHR-CO or NH-CHR-CH_2-CO), i.e. exchange of α- by β-amino-acid residues.
(g) Depsipeptide analogues (NH replaced by O).
(h) Other changes of α-amino groups, e.g. hydrazino (NH replaced by NH-NH), aminoxy (NH replaced by NHO), and N-amino[NH replaced by N(NH_2)] analogues.

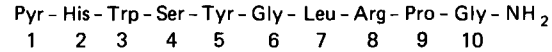
I have selected certain of these modifications for discussion, and shall draw on examples in their application to six bioactive peptides, i.e. thyrotropin releasing hormone (TRH), cholecystokinin/gastrin C-terminal tetrapeptide amide (G-4), the enkephalins, gonadotropin releasing hormone (GnRH), somatostatin (SS), and β-endorphin, the structures of which are shown in Fig. 2.

As regards 'activity', each of the six peptides can elicit a range of biological effects. TRH releases thyrotropin and prolactin, and has CNS effects; G-4 stimulates gastric acid and pancreatic secretion, has various effects on intestinal tone and motility, and may have an important role in the CNS; the enkephalins and β-endorphins exhibit most opiate-like activities and have specific behavioural effects; GnRH is primarily recognised as a releaser of gonadotropins from the pituitary, but it has extra-pituitary effects; SS inhibits, inter alia, the release of growth hormone, insulin, glucagon, and gastric acid secretion.

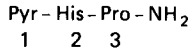
My discussion will centre on the effect of structural change on four aspects of these 'activities', i.e. potency (in respect of one or more of the 'activities' listed), protracted action (likewise), selectivity (between the various activities listed), and the generation of competitive inhibition. The reader is referred to separate reviews of GnRH (Coy and co-workers, 1976; Rivier and co-workers, 1976), TRH (Veber and co-workers, 1976a), SS (Rivier and co-workers, 1976), enkephalins (Morley, 1980a), endorphins (Morley, 1980b), and G-4 (Morley, 1968), and to the excellent annual reviews of S-A relationships (Sheppard 1975-79), for details of the original literature. References are given in the present review only when they are not included in these sources.

CONTRACTION OF CHAIN

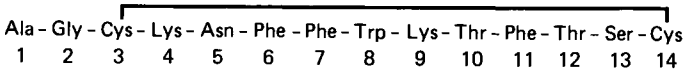
A favoured pursuit with bioactive peptides is examination of shortened sequences derived by N- or C-terminal removal of amino-acid residues, or by deletions within the chain. Where receptors are involved, such sequences may retain all or part of the message transmitted by the parent peptide. This is clearly the case for all six peptides with the exception of TRH. Shortened sequences containing some or all of the parent activities are derived by (a) C-terminal deletions, e.g. β-endorphin⟶



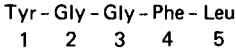
Gonadotropin releasing hormone (GnRH)



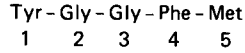
Thyrotropin releasing hormone (TRH)



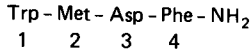
Somatostatin (SS)



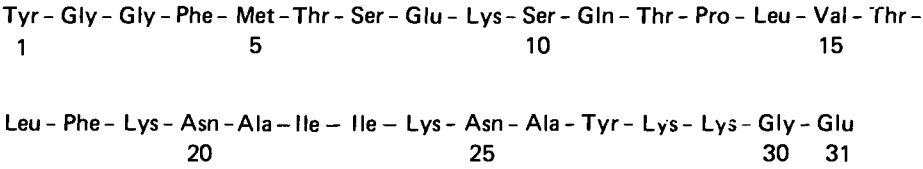
[Leu]enkephalin



[Leu]enkephalin



Gastrin/Cholecystokinin C-terminal tetrapeptide amide (G-4)



β-Endorphin (human sequence)

Fig. 2. Bioactive peptides discussed in this review

[Met]enkephalin, GnRH ⟶ (1-9)-nonapeptide amide, enkephalins ⟶ (1-4)-tetrapeptide; (b) N-terminal deletions, e.g. somatostatin ⟶ (3-14)-dodecapeptide, G-4 ⟶ (2-4)-tripeptide amide); or (c) internal deletions, e.g. SS ⟶ des-Asn^5 analogue, β-endorphin ⟶ (1-5)-(16-31)-eicosapeptide. Potency is usually reduced, but not always so, e.g. [Met]enkephalin is ~3 times more potent than β-endorphin in the mouse vas deferens assay, and GnRH-(1-9)-nonapeptide ethyl amide is considerably more potent than GnRH in most assays.

Selectivity in action may also be seen in shortened sequences. For example β-endorphin 1-16 (=α-endorphin) has opposite behavioural effects to the 1-17 sequence (= ɣ -endorphin), and des-Asn^5 -SS inhibits growth hormone and insulin release, but has little effect on glucagon release. If different types of receptors are involved in the different actions, perhaps the message in the shortened sequence is received by one type, but not another.

The only well established case of a deletion providing an antagonist of the action of the parent peptide is that of des-His^2 -GnRH. Certain derivatives of Trp-Met-Gly have been claimed to antagonise the action of G-4, but the effect is, at best, very weak. These, and other results are summarised in Fig. 3.

Peptide	No. residues	Retained agonism	Antagonism	Selectivity	Protracted action
TRH	3	X	X	X	X
G – 4	4	(√)	(√)	X	X
Enkephalins	5	√	X	X	O
GnRH	10	√	√	X	O
Somatostatin	14	√	X	(√)	X
β – Endorphin	31	√	X	√	X

Fig. 3. Summary of results with analogues of six bioactive peptides derived by contraction of the chain length. A tick (√) implies at least one known example where the activity in question (retained agonism, antagonism, selectivity, or protracted action) has been observed; a cross (X) implies at least one known example, and all examples examined for the activity in question have been found to be without effect; a zero (O) implies no known examples, or known examples have not been examined for the activity in question. Entries in parenthesis indicate doubt as to the significance of results, or very weak effects.

EXTENSION OF CHAIN

Extensions at the N-terminus of peptides may give rise to selectivity. A notable example is seen in the change from G-4 to CCK-like peptides. Thus, G-4 is a potent

stimulator of gastric acid secretion but a relatively poor stimulator of gall bladder secretion; N-terminal addition of O-sulphated Tyr at a critical distance ($\sim$ 2 amino-acid residues) from the amino (Trp)-terminus provides analogues which are poor stimulators of acid, but potent stimulators of gall bladder secretion. C-Terminal extensions may also modify activity as seen in the transitions enkephalin $\longrightarrow$ α- $\rightarrow$ γ- $\longrightarrow$ β-endorphin.

The most fruitful application, however, has been in the design of pro-drug or 'hormogen-like' molecules which have protracted action. Early work showed that Gly-, Leu-, Pro- and Leu-Gly-Gly-oxytocin have consistently higher potencies in vivo than in vitro, and their effects in vivo develop and decay more slowly than the responses to oxytocin (Rudinger, 1971). More recently, similar effects have been seen in N-terminally extended enkephalins. However, N-terminally extended somatostatins are not long-acting in the rat.

Results are summarised in Fig. 4.

Peptide	No. residues	Increased potency	Antagonism	Selectivity	Protracted action
TRH	3	X	X	X	O
G – 4	4	√	X	√	√
Enkephalins	5	√	X	√	√
GnRH	10	X	X	X	O
Somatostatin	14	X	X	X	X
β – Endorphin	31	X	X	X	O

Fig. 4. Summary of results with analogues of six bioactive peptides derived by extension of the chain length (see footnote to Fig. 3 for explanation of symbols).

MODIFICATION OF SIDE CHAINS

Many examples are to be found where structural modification of a side chain leads to analogues with retained or increased potency. The change of Met/Leu^5 in the enkephalins to Pro^5 (resulting in a large increase in in vivo, but not in vitro potency), Gly^6 in GnRH to D-Trp^6 or D-Ser(Bu^t), and Trp in G-4 to Trp(5-Me), may be cited. Antagonists seldom arise, though an exception is [D-Phe^2]-GnRH (His^2 changed) and related GnRH antagonists with additional modifications at the 1,3, and 6 positions.

The best documented case of side chain modification leading to selectivity of action is that of TRH, where dissociation of pituitary (release of TSH) and CNS effects has been achieved. Systematic modification of each of the three amino-

acid residues showed that (a) increasing the ring size of Pyr to a 6-membered ring resulted in a selective increase in the central effects, (b) substitution at the γ position of the imidazole (His) ring by hydrophobic substituents led to selective enhancement of the pituitary effects, while similar substitution by charged substituents led to a selective decrease in the pituitary effects, (c) thia-substitution in the Pro ring led to enhancement of the central and reduction of the pituitary effects, (d) replacement of the C-terminal amide by methyl ester resulted in selective reduction of the pituitary effects. Maximal dissociation (35-fold increase in the central effect, no increase in the pituitary effect) was obtained in an analogue in which 'desirable' features were combined (Fig. 5).

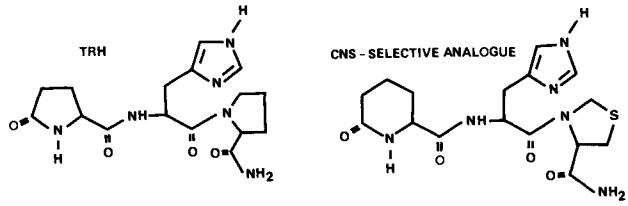


Fig. 5. Structures of TRH and CNS-selective analogue

Results are summarised in Fig. 6.

Peptide	No. residues	Increased potency	Antagonism	Selectivity	Protracted action
TRH	3	√	X	√	X
G – 4	4	√	(√)	X	X
Enkephalins	5	√	X	(√)	X
GnRH	10	√	√	X	X
Somatostatin	14	X	X	√	X
β – Endorphin	31	(√)	X	X	X

Fig. 6. Summary of results with analogues of six bioactive peptides derived by modification of one or more side chains (see footnote to Fig. 3 for explanation of symbols).

D-SUBSTITUTION

Substitution of glycyl or L-amino-acid residues by D-amino acids at appropriate sites of some bioactive peptides may cause a considerable rise in potency. The effect is most dramatic at the Gly^6 position of GnRH, the Gly^2 position of enkephalins, and the Trp^8 position of SS. In the first two cases, many D-substitutions

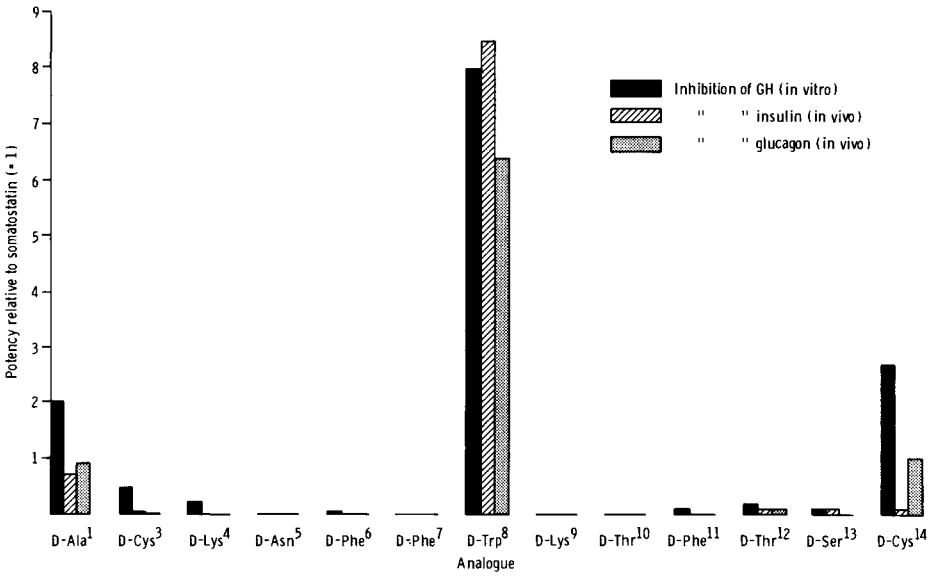


Fig. 7. Potencies, relative to somatostatin = 1, of somatostatin analogues derived by substitution of each L-amino-acid residue by corresponding D-amino-acid residues. For each analogue, three columns are given representing potencies in the following tests: inhibition, in vitro, of spontaneous secretion of growth hormone (GH) by primary cultures of enzymically dispersed rat anterior pituitary cells (first dark column), and inhibition, in vivo of arginine-stimulated release of insulin (second cross-hatched column) or glucagon (third stippled column) (analogues administered intravenously in rats). The D-Asn^{5}, D-Phe^{7}, D-Lys^{9}, and D-Thr^{10} analogues are inactive in all three tests. Of the other analogues, [D-Trp]^{8}-somatostatin stands out as the most potent in all tests. Note also the selectivity seen with [D-Cys]^{14}-somatostatin (Data from Rivier and co-workers, 1976).

have been studied and most cause increased potency. With GnRH, the effect is probably maximal in the D-Trp^{6} (36 times more potent than GnRH in a typical *in vitro* assay) or D-Ser(Bu^{t})^{6} analogues; a rough order of potency for other 6-substituted analogues is D-Tyr > D-Phe > D-Arg > D-Lys > D-Ala > D-Leu > D-Met. With [Leu]enkephalin, the effect in the guinea pig ileum assay is maximal in the D-Ser^{2} analogue (55 times more potent than [Leu]enkephalin); the order of potency of other 2-substituted analogues is D-Ala > D-Thr > D-Met > D-Phe > D-Leu > D-Asp (all more potent than [Leu]enkephalin) > D-Lys > D-Trp (both less potent than [Leu]enkephalin). With [Met]enkephalin, the effect of D-substitution at position 2 is less pronounced. [D-Trp]^{8}-SS is 8 times more potent than SS in most *in vitro* assays, but the effect of other D-substitutions at position 8 has been little studied; [D-Tyr^{8}]-SS is only weakly active, and [D-Ala^{8}]-SS is inactive. Systematic replacement of other amino-acid residues by D-residues in GnRH, enkephalins and SS, and of all residues in G4, TRH and tachykinins has usually provided inactive or weakly-active analogues. Exceptions are the modest increases in potency seen with [D-Ala^{1}]-SS, [D-Ala^{2}]-SS, and in certain assays, [D-Cys^{14}]-SS, and [D-Leu^{5}]-enkephalins. The results with SS are summarised in Fig. 7. D-substitution is thus a useful means of achieving increased potency, but only in certain peptides, and then at specific sites.

When D-substitution is applied at the N-terminal portion of GnRH, competitive antagonists of the pituitary actions of GnRH arise. In the design of these antagonists the key step is replacement of the His^{2} residue by certain D-amino-acid residues, notably D-Phe. Combination of this change with other D-substitutions gives rise to the most potent known antagonists of any bioactive peptide. Early results led to [D-Pyr^{1}, D-Phe^{2}, D-Trp^{3,6}]-GnRH, which blocks ovulation by 100% at a dose of 250μg rat, inhibits GnRH mediated LH secretion by 50% at a concentration ratio (ICR_{50}) of 3:1 (antagonist:GnRH), and can terminate pregnancy when given post-coitally in rats Coy and co-workers (1979) have described [Ac-D-Phe^{1}, D-Phe(p-Cl)^{2}, D-Trp^{3,6}]-GnRH, which blocks ovulation by 100% at a dose of 62μg/rat, and workers at the Salk Institute have an analogue which blocks ovulation by 100% at 20μg/rat, inhibits at ICR_{50} *in vitro* of 0.05.1, and has 25 times higher affinity for the GnRH pituitary receptor than does GnRH. Despite considerable work with the enkephalins, G-4, TRH, and many other peptides, D-substitution has either not provided antagonists, or has provided antagonists of doubtful character, e.g. we have not been able to substantiate the claims for [D-Ala^{4}]-G-4 (Searle and Co., 1977).

There is at least one case where D-substitution has led to selectivity of action. As compared with SS, [D-Cys^{14}]-SS is a poor inhibitor of insulin, but a potent inhibitor of GH and glucagon secretion (see Fig. 7).

Finally, whilst the above D-substitutions are also associated with increased stability towards known proteolytic enzymes, body fluids or tissue extracts,

resulting analogues seldom show protracted effects. A possible reason for this apparent discrepancy is forthcoming from mathematical analysis of a three-compartment pharmacokinetic model (Pliška, 1968); in certain situations only changes in inactivation within the receptor compartment will affect the time course of a response, whereas changes in other compartments may cause an increase in the intensity of the response without appreciable change in the time course. Thus, specific membrane-bound deactivating enzymes may have a critical role in determining protracted action, and the effect of structural change in conferring stability towards such enzymes may not be properly assessed in model experiments with known proteolytic enzymes, body fluids or tissue extracts.

Results are summarised in Fig. 8.

Peptide	No. residues	Increased potency	Antagonism	Selectivity	Protracted action
TRH	3	X	X	X	X
G−4	4	X	?	X	X
Enkephalins	5	√	X	√	√
GnRH	10	√	√	X	O
Somatostatin	14	√	X	√	X
β − Endorphin	31	(√)	X	(√)	X

Fig. 8. Summary of results with analogues of six bioactive peptides derived by substitution of a glycyl or L-amino-acid residue by a D-amino-acid residue (see footnote to Fig. 3 for explanation of symbols; the question mark implies unsubstantiated claims).

α-AZA-SUBSTITUTION

α-Aza analogues of G-4, the enkephalins, GnRH and somatostatin have been described, but not of TRH and β-endorphin (see Dutta and Morley 1976, and Hirst and co-workers 1980).

Substitution of Gly by Azgly has provided particularly fruitful results; [Azgly^{3}]-[Leu]enkephalin is 4 times more potent than [Leu]enkephalin, and [Azgly^{10}]-GnRH is considerably more potent than GnRH. At locations where D-substitution gives rise to increased potency, α-aza-substitution may cause quantitatively similar effects, e.g. [D-Ala^{2}]- and [Azala^{2}]-[Leu]enkephalin are equipotent; however, [Azala^{6}]-GnRH is less potent than [D-Ala^{6}]-GnRH. The Azasp^{2}- and Azphe^{4}-analogues of G-4, and Azphe^{6}-analogues of SS are inactive, as are corresponding D-Asp^{2}, D-Phe^{4} and D-Phe^{6} analogues.

Although α-aza substitution confers considerable stability to the action of important proteolytic enzymes, protracted action was not seen in one GnRH analogue examined. However, Niedrich and co-workers (1969) found that [Azasn^5]-eledoisin-(4-11)-octapeptide had 4-5 times greater duration of action than the parent octapeptide.

Results are summarised in Fig. 9.

Peptide	No. residues	Increased potency	Antagonism	Selectivity	Protracted action
TRH	3	O	O	O	O
G – 4	4	X	X	X	X
Enkephalins	5	√	X	X	O
GnRH	10	√	X	O	X
Somatostatin	14	X	X	X	O
β – Endorphin	31	O	O	O	O

Fig. 9. Summary of results with analogues of six bioactive peptides derived by substitution of a glycyl or L-amino-acid residue by an α-aza-amino-acid residue (see footnote to Fig. 3 for explanation of symbols).

CARBA SUBSTITUTION

There have been two recent developments of interest. First, replacement of the Tyr-Gly bond of enkephalins by CH_2-CH_2, CH_2-NH, or trans CH=CH has provided active analogues. In receptor binding assays, the potencies of the double bond and CH_2-NH analogues are comparable and at least half that of the parent enkephalins, whilst that of the CH_2-CH_2 analogue is much reduced (Hann and co-workers 1980; Hudson and co-workers 1980). Second, replacement of the S-S bridge of somatostatin analogues by CH_2-CH_2 or CO-NH has provided analogues with protracted action and, in the case of the CO-NH bridged analogues, with selectivity for growth hormone inhibition (Garsky and co-workers 1976; Grant and co-workers 1976; Veber and co-workers 1976b).

OTHER BACKBONE MODIFICATIONS

Retro-enantiomers constructed from D-amino-acid residues bear a close topochemical relationship to the parent peptide and differ mainly in the reversal of peptide bonds (see Veber 1979 for reservations about the 'fit'). Several such retro-enantiomers of bioactive peptides (including G-4), in which all peptide bonds are reversed, have been prepared and found inactive (Hayward and Morley, 1974). Partial retro-enantiomers of TRH, GnRH and the enkephalins, in which one or two of the peptide bonds are reversed, have also been prepared by Chorev and co-workers (1979).

When the reversal is at the Phe-Leu/Met bond of the enkephalins (a bond probably not involved in the opiate receptor interaction), potent analogues with long lasting effects resulted. Other analogues were almost inactive.

Kisfaludy and his colleagues have described 36 analogues of Boc-β-Ala-G-4 (= pentagastrin) derived by replacement of α-amino groups of individual amino-acid residues by α-aminoxy. As compared with pentagastrin, several involving aminoxy replacement at the N-terminal residue, provoked increased gastric secretory response and were better absorbed intrajejunally (Schön and co-workers, 1978).

Methylenethio replacement has so far been applied to only one of the six peptides being discussed. Because the Gly-Leu bond in GnRH is very vulnerable to proteolysis, Spatola and co-workers (1979) replaced this bond by CH_2-S in GnRH and a known GnRH antagonist. The potency of resulting analogues, presumably now more resistant to proteolysis, was nevertheless markedly reduced as compared with the parent peptides. The results were attributed as a consequence of the introduction of unacceptable flexibility in a portion of the molecules where conformational flexibility may be essential.

CONCLUSION

There are exciting prospects for the future in correlating peptide and non-peptide structure/conformation, and applying the knowledge in drug design. A major problem in these correlations is that the relevant conformation of the peptides is that adopted at the receptor, and work with the enkephalins and other bioactive peptides indicates that this is not related to preferred conformations in the solid state, or in solution. New approaches are clearly needed before progress can be made.

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Chemical and Biological Properties of Beta-Lipotropin and Endorphins: Metabolism and Mechanisms of Inactivation

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ABSTRACT

A review of the present day knowledge on the biosynthesis, mode of action, metabolism, inactivation and the use of synthetic analogues of the endogenous opiate peptides (EOP) shows remarkable progress in a relatively short time. Two independent endorphin (or EOP) systems have been defined, one with two pentapeptides (methionine and leucine-enkephalin) as mediators and the beta-lipotropin/beta endorphin system of larger peptides. The two main areas discussed in this review are those of inactivation and the recently introduced synthetic analogues of enkephalins. Our own studies have shownthat complex enzymatic systems in different tissues and serum are involved in the inactivation of EOP, both by the rates of degradation of the peptides as well as by the use of non-specific peptidase inhibitors. In two experimental models (the guinea pig ileum preparation and the rat brain opiate receptor) we could correlate the relative potency of enkephalin analogues with their rate of enzymatic inactivation.

KEYWORDS

Endogenous opiate peptides; endorphin inactivation; peptidase inhibitors; enkephalin analogues.

INTRODUCTION

In 1975, several groups of investigators were able to demonstrate that extracts from brain and pituitary contained opiate-like activity (Terenius and Wahlstrom, 1975; Hughes, 1975; Pasternak and co-workers, 1975; Cox and co-workers, 1975). These substances were identified as opiates by their pharmacological effects on smooth muscle bioassays (Hughes and co-workers, 1975). Hughes and co-workers (1975 characterized the brain opiate-like material, demonstrating that it was composed of a mixture of two pentapeptides that were named methionine and leucine-enkephalin (Met-E and Leu-E). Other peptides with opiate-like activity were subsequently found in different parts of the central nervous system (CNS) and the generic term "endorphin" was introduced (see Beaumont and Hughes, 1979 and Snyder and Innis, 1979 for reviews). Soon after the characterization of the enkephalins, beta-lipotropin (B-LPH), a pituitary hormone composed of 91 aminoacid residues (Li, 1964), was shown to have a 61-91 fragment with opiate-like activity; this fragment was

then called "C fragment of B-LPH" or "beta-endorphin" (B-END) (Bradbury and co-workers, 1976; Cox and co-workers, 1976). In addition, the 61-65 aminoacid sequence of B-LPH was recognized as that of Met-E. Thus, B-LPH gained considerable attention in the last years, mainly as the possible precursor of other neuropeptides with biological activity such as beta-MSH (B-LPH 41-58) and B-END (B-LPH 61-91). Unfortunately, the exact functional role of this molecule in the pituitary, as well as its relationship with the different endorphins that it may generate, remains still undefined.

"Endorphin" is accepted as a general term for endogenous opiate peptides (EOP). However, this term may actually create confusion, since there are several endorphins (such as beta, gamma, delta endorphins) that are considered specific and, in addition, such terminology would probably exclude other non-peptide endogenous opiate substances (Gintzler and co-workers, 1976). However, in this paper, we will use EOP and endorphins as equivalent terms.

At present two independent EOP systems have been defined: one in the brain and peripheral nervous tissues with Met-E and Leu-E as mediators and the B-LPH/B-END system which predominates in the pituitary. Several studies have demonstrated that the enkephalins are not metabolites nor artifacts formed during tissue extraction (Hughes and co-workers, 1977; Yang and co-workers, 1977) and that they do not seem to be derived from B-LPH, forming a separate endorphinergic system found in different neurons. Recent immunohystochemical studies have demonstrated the existence of separate enkephalin and B-END containing areas in the brain (Bloom and co-workers, 1978a). Enkephalin-containing cells and nerve terminals are widely distributed in brain and spinal cord, with the highest concentrations found in globus pallidus, hypothalamus, striatum, brainstem and dorsal horn of the spinal cord (Hokfelt and co-workers, 1977; Watson and co-workers, 1978). Most of the enkephalin-containing neurons appear to be short interneurons and recent studies have also shown that Met and Leu-E occur in separate nerve populations (Larsson and co-workers, 1979). The functional or physiological significance of such compartamentalization is still unknown. In contrast, there appears to be only a single B-LPH/B-END cell group located mainly in the arcuate region of the brain, with long axons reaching midbrain and limbic structures (Bloom and co-workers, 1978b); Watson and Barchas, 1979). In the pituitary, the distribution of B-END and enkephalins has been recently described by Rossier and co-workers (1979, 1980). By immunohystochemical and radioimmunological methods, these authors showed that while B-END is present in adenohypohysis and pars intermedia, the enkephalins are found exclusively and in high concentrations in neurohypophysis, suggesting that the pentapeptides may modulate neurohypophyseal secretion.

In addition to their presence in the CNS and hypohysis, EOP have been also found in peripheral tissues such as the gastrointestinal tract (Hughes and co-workers, 1977; Polak and co-workers, 1977; Linnoila and co-workers, 1978; Musacchio and co-workers, 1980) various autonomic nerves and ganglion cells (Hughes and co-workers, 1977), the adrenal gland (Viveros and co-workers, 1979), blood (Wardlow and Frantz, 1979; Hollt and co-workers, 1979; Clement-Jones and co-workers, 1980) and probably placenta (Nakai and co-workers, 1978; Juillard and co-workers, 1980).

Little is known of the possible physiological roles and pharmacological effects of endorphins. They appear to be related to a wide variety of physiological functions such as sensory transmission, endocrine control, behaviour, regulation of respiration, blood pressure, motor activity, body temperature, gastrointestinal function, etc. Endorphins may be related also to several pathological states such as mental illness (schizophrenia and depression), drug addiction, convulsive disorders, asthma, shock, etc. As implied from this list of multiple effects of the EOP, a definite role for the endorphins in normal and pathological states remains to be established.

Most of the assumptions on the physiological and pathological roles of the

endorphins are based on their pharmacological effects in various in vivo and in vitro assay systems, with the expectation that the pharmacological response may simulate the physiological events. Thus, it seems important to point out that, in general, the pharmacological studies with exogenous administration of endorphins are complicated by many factors: 1) the complexity and diversity of the systems in which endorphins are involved; 2) the lack of specific and sensitive assays; 3) the multiplicity of opiate receptors, together with the lack of knowledge about their specific anatomical site and the proportion of different receptors at one anatomical site; 4) the difficulty of access of the administered peptides to their site of action depending on route of administration and 5) the rapid metabolism and relative instability of the natural peptides.

It is highly probable that EOP may turn out to be important neuromodulators or transmitters for complex physiological functions. However, it is also apparent that extreme care must be applied, before drawing conclusions from these type of pharmacological experiments, regardless of how supportive the results may be for an apparent physiological role for the EOP.

This paper will be a cursory update of the field, concentrating mostly on EOP inactivation and recent findings on synthetic analogues of endorphins.

BIOSYNTHESIS

There is now direct evidence that enkephalins and B-END are derived from the breakdown of larger inactive precursor molecules (Mains and co-workers, 1977; Rubinstein and co-workers, 1978; Huang and co-workers, 1979; Kimura and co-workers, 1980). Apparently there is no direct relationship between B-END and the enkephalin precursor molecules, a fact that further supports the view that there are at least two separate and probably independent EOP systems in vertebrates. In general terms, the synthesis of EOP occurs from the breakdown of biologically inactive larger precursors via cleavage by intracellular ensymes, producing the smaller active peptides.

B-END is formed by enzymatic cleavage of the Arg^{60}-Tyr^{61} bond of B-LPH, by a trypsin-like enzyme, (Graf and co-workers, 1978) and pulse labelling experiments with radioactive aminoacids produced labelled B-LPH as well as B-END (Crine and co-workers, 1977). In addition, adrenocorticotropic hormone (ACTH) and B-END originate from a common precursor, a larger molecular weight protein (31,000) which has been termed "pro-opiocortin" (Mains and co-workers, 1977; Rubinstein and co-workers, 1978). Furthermore, both ACTH and B-END appear to be contained in the same secretory granules (Bloom and co-workers, 1978b) and are also released simultaneously (Guillemin and co-workers, 1977).

The evidence that enkephalins are formed from the breakdown of larger precursors is also well established. This evidence originates from studies that followed the incorporation of labelled ^{3}H-tyrosine into the pentapeptides after intraventricular administration (Yang and co-workers, 1978) or after incubation in presence of isolated preparations of guinea pig ileum or slices of striatum (Sosa and co-workers, 1977; Hughes and co-workers, 1978). The incorporation was greatly reduced by puromycin or cycloheximide, indicating that the first step in the biosynthesis of enkephalins is also ribosomal synthesis of a larger precursor, (Hughes and co-workers, 1978). Recent reports suggest that Leu-E may originate from a larger precursor molecule termed alpha-neo-endorphin, at least using hypothalamic extracts (Kangawa and co-workers, 1979). Similarly, a pro-Met-E has been described by Huang and co-workers (1979), from porcine hypothalamus. A most interesting family of enkephalin precursor peptides have been obtained from adrenal gland by Kimura and co-workers (1980), with the remarkable feature that one single large peptide seems to be the precursor for both Met-E and Leu-E. It is probable that comparable precursors could occur in CNS and other tissues involved in enkephalin production.

If we accept the above facts on the bio-synthesis of EOP, it becomes crucial to actually define the complete sequence of steps involved in the process of biosynthesis, storage, release, mode of action and innactivation.

RELEASE AND MODE OF ACTION

Endorphin release has been reported both *in vivo* and *in vitro* by several groups of investigators using different systems. Smith and co-workers (1976) showed a potassium-evoked-calcium-dependent release of enkephalins from rat brain synaptosomes. Similar results were obtained using slices of striatum and globus pallidus (Henderson and co-workers, 1978; Osborne and co-workers, 1978). We have reported an endorphine release induced by electrical stimulation of the guinea pig myenteric plexus longitudinal muscle (MPLM) preparation (Puig and co-workers, 1977a; Puig and co-workers, 1977b, 1978), although we have yet been unable to identify the EOP released by high frequency electrical stimulation. Recently, Viveros and co-workers (1980) have reported a Ca-dependent secretion of enkephalins from perfused adrenal glands. *In vivo* studies have shown that focal brain stimulation in humans releases endorphins into the cerebro-spinal fluid (Akil and co-workers, 1978), suggesting a direct link between the release of enkephalin-like substances and stimulation-produced analgesia.

The release of immunoreactive B-END has been obtained *in vitro* from cultured pituitary tumor cells (Simantov, 1978), isolated pituitary lobes (Przewlock and co-workers, 1978) and from rat hypotalamic slices (Osborne and co-workers, 1979). *In vivo* studies have shown the release from pituitary into blood of immunoreactive B-END in response to stress in rats (Guillemin and co-workers, 1977). Although there is some suggestive evidence that vasopressin may be a physiological releasing factor for ACTH, and by implication or B-END, (Lowry and co-workers, 1980), this is an area that needs intensive study.

There is solid evidence that opiates exert their biological effects through specific membrane binding sites (receptors). It is accepted that at least three families or classes of opiate receptors may be present in the nervous tissues of vertebrates: namely the mu, kappa and delta receptors described in chronic spinal dogs and in different isolated organs (Martin and co-workers, 1976; Lord and co-workers, 1977; Kosterlitz and co-workers, 1980). These receptors have been defined by the affinity of the ligands that predominantly bind to them: morphine (mu) ketazocine-like drugs (kappa) and less defined EOP (delta). The discovery of the EOP and their synthetic analogues has revealed new aspects as well as new problems concerning the interaction of the opiates with their specific receptors. At present very little is known about the nature and characteristics of the binding sites and about the conformation and configurational changes of the molecules involved. The concentrations of different opiate receptors varies within species and organs, and from a practical standpoint, these properties have been used to develop bioassays that serve both the purposes of receptor analysis as well as to study the effects of new opiates or synthetic analogues. Two of such assays have been widely used: the guinea pig ileum preparation (GPI) which has mainly mu-receptors and the mouse vas deferens (MVD) with preferential expression of delta-receptors. These bioassays, together with binding assays with radiolabelled ligands (for example naltrexone for mu and Leu-E for delta) have enabled Kosterlitz and co-workers (1980) to predict the analgesic potency of opiates based on the mu/delta ratios. Opiate peptides with GPI/MVD ratio of 1 or higher, will probably have analgesic activity, while peptides that are more potent agonists in MVD than GPI may have not. Therefore, it is highly probable that mu-receptors are of particular importance in pain perception and analgesia (Law and Loh, 1978; Kosterlitz and co-workers, 1980).

Regardless of receptor heterogeneity and differences in EOP properties, once the opiate (whether endogenous or exogenous) binds to its receptor, the mechanism of

action appears to be similar. In all instances the inter-action appears to produce the inhibition of the release of other neuro-mediators. This has been demonstrated in peripheral nervous system for acetylcholine and noradrenaline (Waterfield and co-workers, 1977) and in CNS for substance P (Jessel and Iversen, 1977), dopamine (Loh and co-workers, 1976a), acetylcholine (Subramanian and co-workers, 1977) and noradrenaline (Taube and co-workers, 1976).

METABOLISM AND INACTIVATION

The three EOP with a probable physiological role (B-END, Met-E, Leu-E) are all inactivated by enzymatic hydrolisis. Two types of enzymatic processes are important in endorphin metabolism: one, as described previously, is the cleavage of active peptides from larger inactive precursors; the other, is the enzymatic process of inactivation of the peptides once they have been generated. Little is known about the former enzymatic process, while a substantial amount of information is being produced, presently, on the latter.

Aminopeptidases, peptidyldipeptidases and caboxypeptidases are probably involved in the catabolism of enkephalins (Shaw and Cook, 1978; Craviso and Musacchio, 1978; Erdos and co-workers, 1978; Malfroy and co-workers, 1979). It is well established that exogenously administered enkephalins are inactivated by cleavage of the Tyr-Gly bond with the consequent release of free Tyr and a residual tetrapeptide devoid of opiate activity in the standard assays (Marks and co-workers, 1977). A different picture may be true when the endogenous peptides are inactivated in situ after being physiologically released. It seems probable that in this case, besides aminopeptidases, some specific enzymes would be responsible for the termination of the action of the enkephalins. Most tissues are known to contain a variety of peptidases capable of enkephalin inactivation. This constitutes a technical difficulty in the determination of the pharmacological effects of exogenously administered enkephalins. One tissue preparation that we have analysed in detail is the GPI (guinea pig ileum) preparation (Puig and co-workers, 1977c; Craviso and Musacchio, 1978; Musacchio and co-workers, 1979). Although the presence of enzymes that inactivate EOP in the GPI was reported (Hughes, 1975), the precise nature and characteristics of the enzymes involved was not known. Our recent work has demonstrated that GPI preparations contain families of peptidases that degrade both Leu-E and Met-E and that the main mechanism of inactivation is the cleavage of the N-terminal bond of the pentapeptides (Craviso and Musacchio, 1978; Musacchio and co-workers, 1979). It is also apparent from our results that other enzymes such as carboxypeptidases may be involved in enkephalin inactivation (Puig and co-workers, 1977c).

The initial experiments designed to study the metabolic inactivation of enkephalins were carried out by incubation of the peptides with GPI preparations (using either MPLM or whole ileum segments) and with a crude rat brain membrane preparation (Puig and co-workers, 1977c). In the ileum experiments the peptides were kept in contact with the biological preparation and the electrically induced contractions constantly monitored, and subsequently used to measure the decrease in activity of the peptides. The time-course of the inhibition curves was compared with a control curve for morphine, a substance that is not affected by the endogenous peptidases. In other experiments the peptides were incubated with the brain membranes and then their morphine-like activity tested in MPLM preparations and in opiate receptor binding assays. Our experiments showed that both the GPI and the crude brain membrane preparations were capable of inactivation of the enkephalins. This inactivation was due to tissue peptidases that destroy the peptides very rapidly, with a half-life of about 15 minutes. It is interesting to point out that Met-E-amide (MetA-E) was more resistant to GPI peptidases than Met-E and Leu-E, however the crude brain membrane preparation was able to inactivate the amide-derivative at the same rate as the naturally occurring pentapeptides. This observation suggested that different enzymatic systems may operate in these two tissue preparations. To clarify these

possible differences, we started studies aimed at enzyme characterization and the screening of adequate enzyme inhibitors. The value of the enzyme inhibitors is three fold: firstly, their capacity to protect the enkephalins from inactivation will permit more acurate pharmacological studies; secondly, they will enable the better study of endogenous enkephalinergic systems and thirdly, they will help to define the inactivating enzymes involved. The characterization of a certain enzyme or its partial purification has to be based on indirect approaches such as the detection and quantification of the breakdown products or the functional evaluation of the effects of the enzyme inhibitors on the system. Using ^{3}H-tyrosine labelled Met-E and Leu-E, we could show that MPLM strips, intact ileum segments, guinea pig serum as well as 100,000 x g supernatant fractions of MPLM strips and brain, contained high levels of peptidase activity (Craviso and Musacchio, 1978; Musacchio and co-workers, 1979). A simple procedure was used: the labelled peptides were incubated with the different tissue preparations; the reaction was interrupted by removal of the tissues and/or acidification; aliquotes of each incubation medium were spotted on silica gel plates and analyzed by thin layer chromatography. From the radioactivity present in the areas corresponding to enkephalin and tyrosine we could account for the inactivation of the enkephalins by cleavage of the Tyr-Gly bond.

Since for all the tissue preparations tested, almost all of the radioactivity could be accounted as free tyrosine, we concluded that the main event in the degradation of enkephalins was the cleavage, probably by aminopeptidases, of the Tyr-Gly bond. It is highly probable that these enzymes are non-specific; obviously, these results do not exclude the presence of specific enkephalin degrading systems, in the tissues studied. Using this same system, Craviso and Musacchio (1978) studied a variety of peptidic and non-peptidic compounds which could act as putative enzyme inhibitors. Of all the compounds tested only O-phenantroline, 8-OH-quinoline and hexachlorophene could completely inhibit enkephalin degradation from all tissue preparations (see above). Other enzyme inhibitors tested such as bacitracin, puromycin and EDTA were very effective inhibitors of the enzymes in the 100,000 x g supernatants and the MPLM leaching enzymes, with relatively little effect on the serum and intact ileum leaching enzymes. These results differentiate at least two groups of enzymes, one mostly derived from disrupted tissues and the other from serum. These differences were further substantiated after Sephadex G-200 gel filtration. Although the elution profile peaks were rather broad in all the preparations (probably due to multiplicity of enzymes), different peaks were obtained for 100,000 x g supernatants and serum enzymatic activities. Based on the effects of the above mentioned inhibitors on the Sephadex fractions obtained from the MPLM leaching enzyme it was observed that such fractions contained both the serum and the disrupted tissue enzymatic activity in early and late fractions. These series of experiments points both to the apparent heterogeneity of the enzymatic systems involved in enkephalin inactivation as well as at the methodological problems derived from these studies.

At present, besides theaminopeptidases that inspecifically cleave the Tyr-Gly bond of the enkephalins, some enzymes that may have a physiological role in the inactivation of the enkephalins have been described. It has been shown recently that membranes obtained from different tissues such as brain, kidney (Marks, 1978) and human endothelial cells (Erdos and co-workers, 1978) contain bound forms of peptidases. Thus, Schwartz and co-workers (1980 ; Malfroy and co-workers, 1978)described a dipeptidyl-carboxypeptidase ("enkephalinase") present in membrane fractions of striatum that specifically cleaves the Gly-Phe bond of enkephalins, inducing the formation of a Tyr-Gly-Gly tripeptide and Phe-Met. This enzyme appears to be different from the well characterized angiotensin-converting enzyme, demonstrated by their different affinities for specific enzyme inhibitors. Moreover, "enkephalinase" seems to be located in similar areas as the opiate receptors and its activity increases after chronic morphine treatment, suggesting that this enzyme could be involved in enkephalinergic transmission (Schwartz and co-workers, 1980). However this enzyme is not completely specific for enkephalins, since it is also capable of releasing the C-terminal dipeptide of other peptides such as insulin. Interestingly,

a wide variety of other peptides including B-END are not affected by this peptidase. Regardless of how attractive the hypothesis for a specific enzyme for enkephalin inactivation may be, we must keep in mind its similarity with angiotensin-converting enzyme and the possibility that both activities may be mediated by the same enzyme. Of less practical importance is the observation by Stern and Marks (1979) that the Gly-Gly dipeptide could be degraded by a "specific" dipeptidase activated by cobalt. It has been known for some time that the replacement of Gly^2 by D-Ala in the enkephalin pentapeptide, leads to analogues that cannot be inactivated or degraded by aminopeptidases (Pert and co-workers, 1976). Thus, such a replacement, by protecting the amino terminal of the molecule produces a three fold increase in the analgesic activity of the peptide. However, if the enkephalin molecule is modified to make it resistant to "enkephalinase", such as protection of the Gly-Phe bond, a 12-fold increase in analgesic potency is observed (Roemer and Pless, 1979). This suggests a more important role for "enkephalinase" in inactivation than the non-specific aminopeptidases. The combination of several of these structural features has led to the synthesis of enkephalin analogues that seem to be protected against enzymatic degradation by both non-specific aminopeptidases as well as "enkephalinase".

B-END is more stable than enkephalins since its aminoacid conformation appears to protect the N-terminal Tyr from hydrolisis. At least four peptides with apparent opiate-like activity are derived from B-LPH: B-LPH 61-91 or B-END; B-LPH 61-79 or delta endorphin (D-END); B-LPH 61-77 or gamma endorphin (G-END) and B-LPH 61-76 or alpha endorphin (A-END). Graf and co-workers (1978) demonstrated that anterior pituitary homogenates contain proteinases capable of splitting the Arg^{60}-Tyr^{61}, Lys^{79}-Asn^{80} and Leu^{77}-Phe^{78} peptide bonds of B-LPH, thus originating the formation of B-END, D-END and G-END. These enzymes are of two different types: while the cleavage of the Arg-Tyr and Lys-Asn bonds are achieved by peptidases that remain in the particle-bound state (i.e. membrane associated), the enzyme that cleaves Leu-Phe appears to be identical with lysosomal cathepsin D (Marks and co-workers, 1980). Based on these studies it is accepted that cathepsins (D and probably B) are the major enzymes that convert B-END to G-END in brain and pituitary. This may represent the first step in the metabolism of B-END. Carboxypeptidases would act then on the newly formed G-END with production of A-END. In addition, both G-END and A-END are extremely susceptible to the action of aminopeptidases that subsequently cleave the N-terminal Tyr, abolishing their opiate-like activity. However, recent findings that G-END and its des-Tyr derivative have opposite behavioural effects than those of B-END and A-END (De Wied and co-workers, 1978), suggests that endorphins of intermediate size may have still undefined physiological roles, not associated with classical opiate-like activity.

Met-E (B-LPH 61-65) has been obtained *in vitro* by incubation of B-LPH with different enzymes, however, it does not seem probable that such a process may occur under physiological conditions.

SYNTHETIC ANALOGUES

Various groups of investigators have exhaustively studied the synthesis and biological activity of several hundreds of enkephalin analogues (Roemer and co-workers, 1977; Pless and co-workers, 1979; Miller and Cuatrecasas, 1979; Audigier and co-workers, 1980). Most of the analogues were synthesized with three main properties in mind: firstly, they should be protected against enzymatic inactivation; secondly, the compounds should have good penetrability and reach their site of action and thirdly, they should preserve a high affinity for the opiate receptor. We have already discussed the problems of enzymatic inactivation and will not repeat them here. Similarly, the multiplicity of receptors, already discussed, is a major factor complicating these type of studies. Thus, modification of the enkephalin molecule may produce analogues with varied affinities for the different populations of opiate

receptors, giving confusing results when their biological activity is tested in different assays (Kosterlitz and co-workers, 1980). Since it is generally accepted that endorphins participate in the modulation of pain perception, some of the most interesting enkephalin analogues are those showing analgesic activity. The main results of these studies can be summarized as follows: Analogues that are more active than morphine or B-END in producing analgesia have been synthesized (Roemer and co-workers, 1977; Wei and co-workers, 1978; Hollosi and co-workers, 1980); some analogues produce analgesia after systematic administration (Tseng and co-workers, 1976; Szekely and co-workers, 1977) even after oral or subcutaneous administration (Roemer and co-workers, 1977; Roemer and Pless, 1979); some tetrapeptides, especially with the D-Ala^2 substitution are also effective analgesics (McGregor and co-workers, 1978; Fujino and co-workers, 1980). It should be mentioned that some analogues that have low analgesic activity after systematic administration, can produce other effects such as changes in behaviour, motor activity, intestinal motility, etc. (Baxter and co-workers, 1977; Miller and co-workers, 1978; Kastin and co-workers, 1979). These last results suggest a disassociation between the analgesic and behavioural effects of these synthetic peptides.

We have investigated the relative potency and the rate of enzymatic inactivation of the naturally occurring enkephalins and of four synthetic analogues: Met-E amide (MetA-E), the S-Benzyl sulfonium analogue of Met-E (SMet-E), the 3-Benzyl-tyrosine analogue of Met-E, (BTMet-E), and the 3-Benzyl-tyrosine analogue of Leu-E (BTLeu-E) (Puig and co-workers, 1977c). The relative potency of the peptides was determined by measuring their effect on the GPI bioassay and also their ability to displace ^{3}H-naltrexone from the brain opiate receptor. Our results showed that Met-E and MetA-E are about two and three times more potent than morphine, while Leu-E and SMet-E are about four times less effective. BTMet-E is fourty times less potent than morphine and the opiate activity of BTLeu-E was negligible. The increase in potency of the MetA-E analogue indicated that this peptide is relatively more resistant to enzymatic degradation than the natural peptides, and also implies that carboxypeptidases are involved in the inactivation of opioid peptides in the GPI.

It is generally accepted that B-END is the most potent and longer acting of the natural opiates; its analgesic effects have been established for different species and routes of administration (Loh and co-workers, 1976b). This greater opiate-like activity may be the result of increased resistance to enzymatic degradation due to the larger size of the peptide as well as to its special conformation. Several B-END analogues have been synthesized; of all, only D-Ala^2 B-END has the same analgesic potency as the parent molecule, the rest losing totally or partially their opiate-like activity. It is interesting to point out that several groups of investigators have demonstrated behavioural effects without analgesia following systematic administration of B-END or D-Ala^2 B-END (Olson and co-workers, 1978; Gorelick and co-workers, 1978). These effects included changes in motor activity, sexual behaviour and other responses typically related to emotionality in animals. B-END and G-END, as well as the des-Tyr derivative of the latter, have been reported to have beneficial effects in patients with schizophrenia (Rivier and co-workers, 1977; Verhoeven and co-workers, 1978; Usdin, 1979). An area of research that will probably be very fruitful in the future, will be the analysis of the "non-opiate" effects of the endogenous opiate peptides.

CONCLUSION

From this overview on the biosynthesis, metabolism and inactivation of the EOP, it is apparent that considerable progress has been made since their discovery in 1975. Since the peptide structure of these substances is well defined, progress in both metabolism and the development of synthetic analogues has been successful. However, the "ideal" non-addictive opiate has not been found yet. On the other hand, a rapidly developing field is that of the non-opiate effects of the endorphins, which is

evolving into a subject as exciting as the early analgesic studies. Hopefully, the future investigations correlating molecular structure and biological activity will lead to the development of new peptides with high and well defined therapeutic values. In addition, this type of analysis of peptide structure and function through chemical modification, will greatly help our understanding of both peptide chemistry and endorphin function.

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Neurohypophysial Hormones and Neurophysins: Structures, Precursors and Evolution

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ABSTRACT

Neurohypophysial hormones and neurophysins can be extracted from posterior pituitary glands bound in non-covalent complexes. The complexes are dissociated in acidic medium and the low molecular weight hormones (Mr = 1,000) are separated from neurophysins (Mr = 10,000) by molecular sieving. From each species, two neurohypophysial hormones are usually isolated by ion-exchange chromatography and two neurophysins (MSEL- and VLDV-neurophysins, named according to the nature of the aminoacids in positions 2, 3, 6 and 7) are purified in the same way.

Studies on about 50 vertebrates, including man, have shown that mammals have vasopressin and oxytocin when non-mammalian species have vasotocin instead of vasopressin and a specific hormone (mesotocin for tetrapods, isotocin for bony fishes, glumitocin, asparqtocin or valitocin for cartilaginous fishes). All the 9 known hormones are nonapeptides with variations only in positions 3, 4 or 8. Recent investigations on American and Australian Metatheria have revealed that, in contrast to placental mammals, marsupials have two pressor hormones. The duplication, which is assumed to have occurred after the divergence from Eutheria, suggests the common origin of American and Australian Metatheria.

Neurophysins are single-chain proteins (95 residues for MSEL and 93 residues for VLDV). MSEL- and VLDV-neurophysins from five and three species, respectively, have been fully sequenced. There are 80% homology between the two neurophysins from a given species, substitutions occurring in the N-terminal (1-9) and C-terminal (75-95) sequences. These distinctive terminal domains, acting as antigenic determinants, can give antibodies monospecific for each type of neurophysins.

Recent research from several laboratories on possible common precursors for neurohypophysial hormones and neurophysins, both by m-RNA directed synthesis and by direct isolation from hypothalamus, has given a strong support for a macromolecular precursor split into vasopressin and a neurophysin.

KEYWORDS

Neuropeptides ; neurohypophysial hormones ; oxytocin ; vasopressin ; neurophysins ; pro-hormones ; hypothalamic precursors ; protein evolution.

INTRODUCTION

Neurohypophysial hormones and neurophysins are biosynthesized in hypothalamus likely as common macromolecular precursors (Sachs et al., 1969 ; Brownstein et al., 1980) split during the transport until neurohypophysis from which they can be extracted associated in non-covalent complexes. Since the discovery, at the beginning of the century, of the pressor, oxytocic, antidiuretic and milk-ejecting activities in posterior pituitary, two opposing views were held about the number of molecules responsible for these activities. According to the unitary conception, defended first by Abel and later by Van Dyke, a single protein displayed all these biologic properties. This so-called "mother-molecule" could be purified only under mild conditions. In contrast the multimolecule conception led first Dudley and finally du Vigneaud, Fromageot and their colleagues to isolate and characterize two active peptides from mammalian glands, the one, oxytocin, possessing oxytocic and milk-ejecting activities, the other, vasopressin, endowed with pressor and antidiuretic activities.

The question arose to identify the physiological forms and to clarify the relationships between the "mother-molecule" and the active peptides. In 1955 we showed that the "mother-molecule" isolated from ox glands was not a pure substance but a non-covalent complex between oxytocin and vasopressin responsible for the activities and an inactive protein for which we have suggested the name "neurophysin" (Acher et al., 1955, 1956). The complex was found in all the mammalian species investigated. It could be dissociated by a variety of procedures and reconstituted from separate components so that the interaction between the hormones and neurophysin seems specific (J. Chauvet et al., 1960). Further studies on neurophysin revealed several components, distinguishable by electrophoresis, which were tentatively called Neurophysin I, II, III etc. according to the mobility to the anode (Hope and Pickup, 1974). The molecular weights of neurophysins, deduced from structural data, are about 10.000 daltons. The complex has an apparent molecular weight of 20.000-25.000 daltons and seems made by a stoichiometric association between oxytocin, vasopressin and neurophysins in a molar ratio 1:1:2. Chemical characterization of neurophysin components has shown that only two types of neurophysins exist in each species, truncated forms giving additional bands in electrophoresis (Acher, 1979). An attractive hypothesis is that one type is bound to oxytocin in a common precursor and the second type is attached to vasopressin in another precursor. In fact neurosecretory granules containing either oxytocin and one type of neurophysin or vasopressin and the second type have been separated (Hope and Pickup, 1974). Recent reviews on neurohypophysial hormones (Acher, 1974, 1980) and neurophysins (Acher, 1979 ; Breslow, 1979 ; Brownstein et al., 1980 ; Pickering and Jones, 1978 ; Seif and Robinson, 1978) have been devoted to the structural, biosynthetic and evolutionary aspects of these neuropeptides. A general synthesis has been attempted (Acher, 1978).

NEUROHYPOPHYSIAL HORMONES

Miniaturization of the Purification

The complex procedure. Whatever the biologic significance of the association between neurophysins and neurohypophysial hormones might be, the use of the complex as a procedure for the purification of active peptides turned out to be very successful (Acher et al., 1958). The procedure is fast and gives high yields. For the first step one precipitates the complex from posterior pituitary extracts by use of sodium chloride ; for the second step, one dissociates the complex by means of trichloroacetic acid, which precipitates only neurophysins but leaves neurohypophysial hormones in the supernatant solution ; for the third step, one separates the two hormones usually present by chromatography on a single ion-exchanger. The complex can be reconstituted (J. Chauvet et al., 1960) ; so, in species in which intrinsic neurophysins do not give high yields for any reason, it is convenient to add beef or horse neurophysin and to operate under the usual conditions. Because the procedure needs only a few hundred mg of posterior pituitary powder, it could be applied not only to mammals but also to small but abundant species such as chicken or frog (1 g represents 1,500 and 10,000 glands, respectively).

The chromatoelectrophoretic procedure. During the last ten years it was often necessary to perform isolation of neurohypophysial hormones with only a few dozens or one hundred glands from small and rare species such as egg-laying mammals or lungfishes, and a more sensitive method was elaborated. In this procedure all the proteins of the posterior pituitary extract are precipitated with trichloroacetic acid or removed by molecular sieving. Then the peptides are separated by chromatoelectrophoresis on paper. With this microtechnique, from 25-50 mg of posterior pituitary powder (10 echidnas for 25 mg, 10 red kangaroos for 50 mg), approximately 0.03-0.10 mg of neurohypophysial hormone can be obtained. Approximately 0.01 mg (10 nmol) are necessary for determining the amino-acid composition and approximately 0.05-0.1 mg (50-100 nmol) for establishing the amino-acid sequence.

By using these procedures successively, we were able to isolate and characterize, over a period of 20 years, the neurohypophysial hormones of about 50 species belonging to six out of seven classes of vertebrates. Seven new neurohypophysial hormones have been chemically characterized in the course of this exploration.

Structures of the Neurohypophysial Hormones

Up to now, ten neurohypophysial hormones have been characterized in vertebrates. The conclusion can be drawn that on the one hand, each species usually has two neurohypophysial peptides, on the other, that the neurohypophysial hormones are evolutionarily very stable because the same hormones are generally found in species belonging to the same class.

Five main groups can clearly be distinguished in vertebrates. These groups are :

1. The mammals (Prototheria, Metatheria and Eutheria) in which

oxytocin and arginine vasopressin have been found. Rarely lysine vasopressin and phenypressin (Phe^2-Arg^8-vasopressin) have been observed.

2. The nonmammalian tetrapods, namely birds, reptiles, amphibians, and lungfishes, in which two different peptides, mesotocin (Ile^8-oxytocin) and arginine vasotocin (Ile^3-vasopressin) have been characterized.

3. The bony fishes, including primitive bony fishes such as Polypterus, in which isotocin (Ser^4-Ile^8-oxytocin) and arginine vasotocin have been identified.

4. The cartilaginous fishes, which show a rather great hormone heterogeneity in contrast to the other classes. Two particular features have to be noted : if cartilaginous fishes have arginine vasotocin like all the other nonmammalian vertebrates, the amount is very small ; the second hormone differs in each subgroup, namely rays, sharks, and chimaeras. In four species of rays, glumitocin (Ser^4-Gln^8-oxytocin) has been characterized. In the spiny dogfish (Squalus acanthias), valitocin (Val^8-oxytocin) and aspargtocin (Asn^4-oxytocin) have been found and it remains to be determined whether both peptides are produced by a single gland or not. In the chimaeras, the ratfish (Hydrolagus colliei) has been examined (Pickering and Heller, 1969), and a peptide having the amino-acid composition of oxytocin has been isolated. The presence of oxytocin in a fish is rather puzzling.

5. The cyclostomes, the most ancient living vertebrates, in which a single hormone, arginine vasotocin has been disclosed.

Figure 1 shows the amino-acid sequences of the nine hormones previously characterized. A tenth hormone, phenypressin (Phe^2-Arg^8-vasopressin), has very recently been characterized in Australian marsupials (Chauvet et al., 1980 a).

	1 2 3 4 5 6 7 8 9	1 2 3 4 5 6 7 8 9
Mammals (except pig)	Cys-Tyr-Ile-*Gln*-Asn-Cys-Pro-*Leu*-Gly-NH_2 (oxytocin)	Cys-Tyr-*Phe*-Gln-Asn-Cys-Pro-*Arg*-Gly-NH_2 (arginine vasopressin)
Pig	Cys-Tyr-Ile-*Gln*-Asn-Cys-Pro-*Leu*-Gly-NH_2 (oxytocin)	Cys-Tyr-*Phe*-Gln-Asn-Cys-Pro-*Lys*-Gly-NH_2 (lysine vasopressin)
Birds, reptiles, amphibians, lungfishes	Cys-Tyr-Ile-*Gln*-Asn-Cys-Pro-*Ile*-Gly-NH_2 (mesotocin)	Cys-Tyr-*Ile*-Gln-Asn-Cys-Pro-*Arg*-Gly-NH_2 (vasotocin)
Bony fishes (paleopterygians and neopterygians)	Cys-Tyr-Ile-*Ser*-Asn-Cys-Pro-*Ile*-Gly-NH_2 (isotocin)	Cys-Tyr-*Ile*-Gln-Asn-Cys-Pro-*Arg*-Gly-NH_2 (vasotocin)
Cartilaginous fishes (rays)	Cys-Tyr-Ile-*Ser*-Asn-Cys-Pro-*Gln*-Gly-NH_2 (glumitocin)	vasotocin (?)
Cartilaginous fishes (sharks)	Cys-Tyr-Ile-*Gln*-Asn-Cys-Pro-*Val*-Gly-NH_2 (valitocin)	vasotocin (?)
	Cys-Tyr-Ile-*Asn*-Asn-Cys-Pro-*Leu*-Gly-NH_2 (aspargtocin)	vasotocin (?)

Fig. 1. Structures of vertebrate neurohypophysial hormones.

Phylogeny of Neurohypophysial Hormones

Six oxytocin-like hormones and four vasopressin-like hormones have been identified. All these peptides have nine amino-acid residues, five of which are invariant.

The oxytocin-like family. In the oxytocin-like family, substitutions occur only in positions 4 and/or 8. In position 4, a polar residue, namely glutamine, serine, or asparagine is always present. In position 8, a nonpolar residue such as leucine, isoleucine or valine is almost always present with the exception of glutamine in glumitocin. It is of interest to note that this latter hormone, in contrast to the five other oxytocin-like peptides, displays a very poor rat oxytocic activity. A hydrophobic residue in position 8 is probably crucial for the specific binding to the rat-uterus receptor.

The vasopressin-like family. Arginine vasopressin was found virtually in all the placental mammals except the pig and some pig-like species in which it is replaced by lysine vasopressin. However in Metatheria two pressor hormones were observed in five species belonging to the Australian family Macropodidae and in two species belonging to the American family Didelphidae (M.T. Chauvet et al., 1980 a, b). Duplication of vasopressin gene seems likely since the two pressor hormones were present in all the individual glands examined. In the red kangaroo and the tammar, one of them was chemically identified as lysine vasopressin (Hurpet et al., 1980). The second hormone has been identified as Phe^2-Arg^8-vasopressin and named phenypressin (M.T. Chauvet et al., 1980 a). Figure 2 shows the separation of the two pressor hormones carried out on individual glands by ion-exchange microchromatography.

Because arginine vasopressin was the only pressor hormone found in a primitive prototherian, echidna, and in most placental mammals, it seems that duplication occurred after the divergence of Metatheria from the Eutheria. It will be of interest to characterize chemically the pressor hormones of American marsupials in order to compare with those of Australian marsupials.

The only pressor peptide found up to now in nonmammalian vertebrates is arginine vasotocin. The change of arginine vasotocin into arginine vasopressin, usually present in mammals, is due to the replacement in position 3 of isoleucine by phenylalanine ; this substitution of an aliphatic residue for an aromatic one, a single interchange between two hydrophobic residues, produces an increase of the rat antidiuretic activity and a loss of the secondary oxytocic activity displayed by vasotocin.

Phylogenetic lines. Figure 3 shows the successive amino-acid substitutions in the two evolutionary lines. It is of interest to note that there is a clear-cut division between nonmammalian tetrapods and mammals. Both neurohypophysial hormones are changed, and this double change may be explained by a specialization for new functions : lactation on the one hand and antidiuresis on the other. The biological significance of the vasopressin duplication in marsupials remains to be clarified.

Figure 4 combines chemical and paleontologic data to trace a hypothetic evolution of the neurohypophysial hormones. If there is a great complexity in cartilaginous fishes, in contrast from bony fishes to

mammals, two evolutionary lines could be distinguished : the oxytocin line with three steps (isotocin, mesotocin, oxytocin) and the vasopressin line with only two steps (arginine vasotocin and vasopressin).

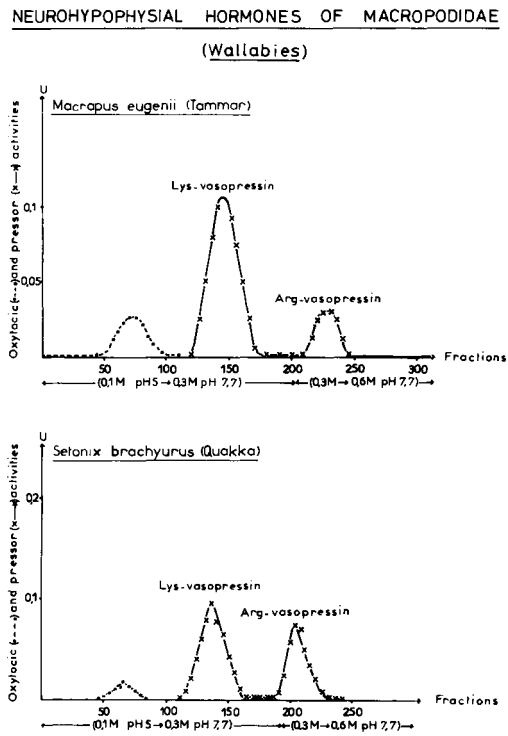


Fig. 2. Duplication of vasopressin in Australian marsupials. (The second vasopressin has now been identified as Phe^2- Arg^8-vasopressin in the tammar)

NEUROPHYSINS

Neurophysins are characterized by the following properties :

1. The ability to give a stoichiometric and reversible pH-dependent complex with the neurohypophysial hormones.
2. An acidic isoelectric point, between pH 4.0 and pH 5.0.
3. A peculiar amino-acid composition, a high content of

proline (8-9 residues), which is unusual in animal proteins, and a low content in aromatic amino acids (a single tyrosine per mole).

4. A very high number of disulfide bridges in the molecule, seven bridges for a polypeptide chain of approximately 100 residues. So the conformation is probably different from those of the known proteins.

The Two Types of Neurophysins

Investigations carried out on seven placental mammals led to the conclusion that despite the number of components varies from one species to another, only two distinct neurophysins exist in each species (Acher, 1979). We have proposed to call these two types MSEL- and VLDV-neurophysins according to the nature of the amino acids in positions 2, 3, 6 and 7. In fact the two neurophysins differ by the N-terminal sequences (residues 1 to 9) and the C-terminal sequences (residues 75 to 95), the central parts (10-74) being nearly identical. The two types of neurophysins can be purified from the complex by ion-exchange chromatography (Acher, 1979).

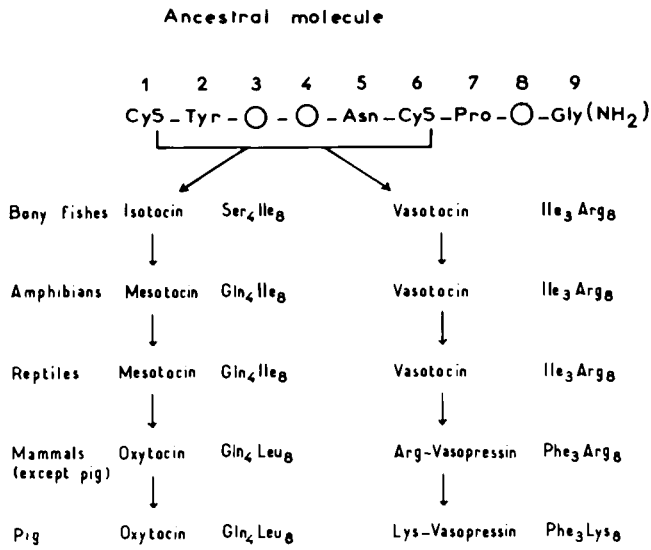
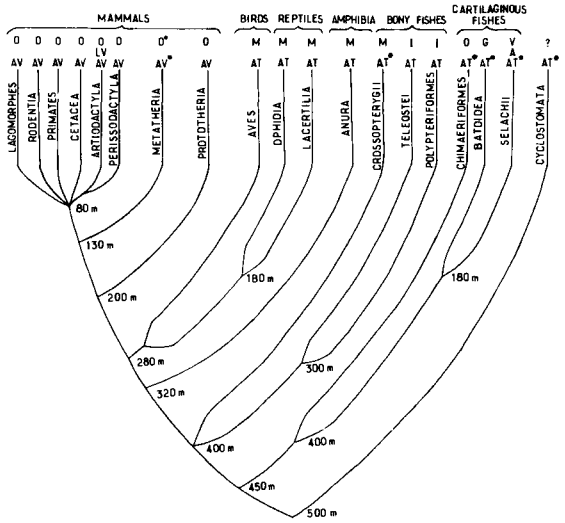


Fig. 3. Hypothetical scheme of evolution of neurohypophysial hormones. One-gene duplication and a series of subsequent single substitutions in positions 3, 4 or 8 produce two molecular lines.



EVOLUTION OF NEUROHYPOPHYSEAL HORMONES

Fig. 4. Neurophypophysial hormones and species evolution according to paleontologic data. Letters indicate hormones identified in modern representatives of groups (asterisk means that identification is not chemical but pharmacologic) : O - oxytocin, AV - arginine vasopressin, LV - lysine vasopressin, M - mesotocin, I - isotocin, A - aspargtocin, V - valitocin, G - glumitocin, AT - arginine vasotocin. Numbers give time in millions of years since divergence.

MSEL-neurophysins. MSEL-neurophysins of five species, namely ox, sheep, pig, horse and whale, have been completely sequenced and those of man and rat are in progress. Figure 5 shows the results for four species.

These neurophysins have 95 residues and variations are virtually observed only in the last seven positions. Within the family the number of substitutions varies from 1 to 5 when the sheep is taken as reference.

VLDV-neurophysins. Amino-acid sequences of the VLDV-neurophysins from three species, namely ox, pig and horse, have been determined. Results for ox and pig are shown in Fig. 6. VLDV-neurophysins have 93 residues. The number of substitutions between ox and pig is 6.

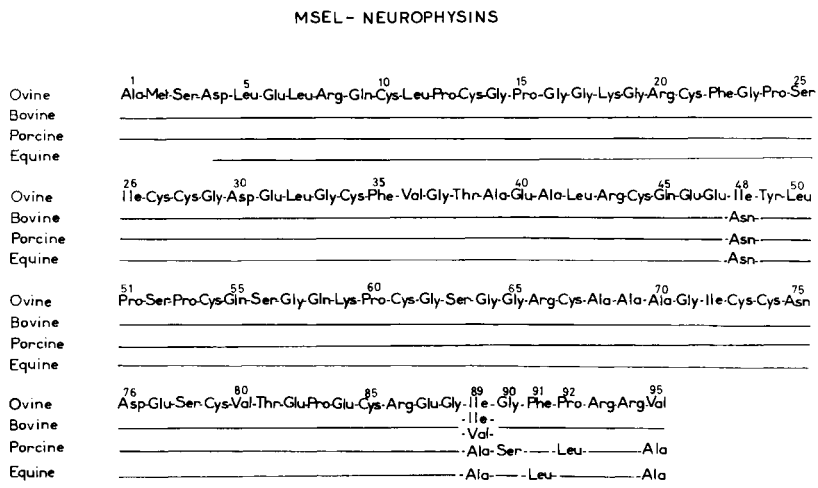


Fig. 5. Amino-acid sequences of some MSEL-neurophysins.

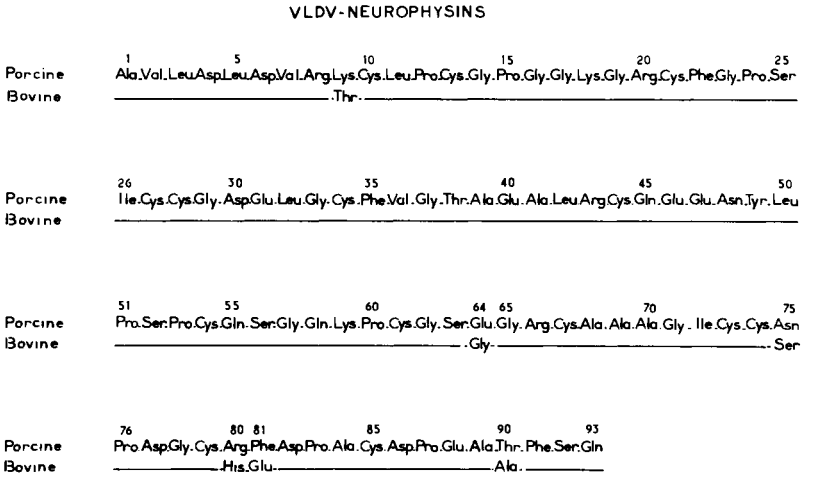


Fig. 6. Amino-acid sequences of two VLDV-neurophysins.

Comparison between the two families is shown in Fig. 7. In a given species, there are about 20 substitutions between the two types of neurophysins. Because these substitutions are concentrated in N- and C-terminal sequences, these regions act as specific antigens and the two types of neurophysins can be distinguished by monospecific antibodies (Robinson, 1975). Half-cystines residues are in the same positions in the two types so that it can be assumed that the 7 disulfide bridges are identical. Because of the strong homology in the sequences (80 %) the general conformations are likely very similar.

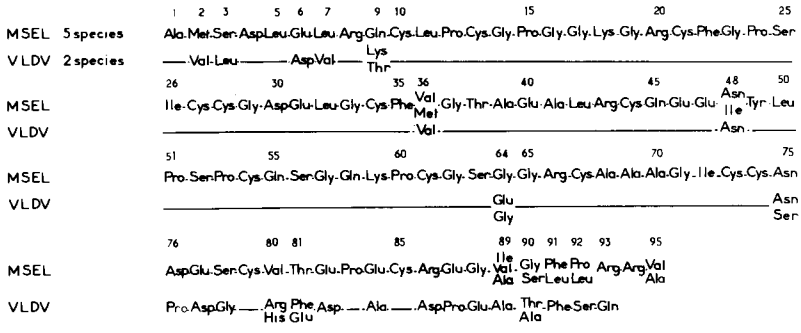


Fig. 7. Comparison between MSEL- and VLDV-neurophysins.

COMMON PRECURSORS

Recent studies on peptide hormones have shown that most of them are manufactured as larger prohormones, that means as a polypeptide chain that is cleaved before secretion into an active shorter peptide and an inactive fragment. For instance, proinsulin, proparathormone, and progastrin have been isolated. It seems reasonable to assume that oxytocin and vasopressin are primarily synthesized as larger molecules. Are neurophysins and hormones cleavage products of common protein precursors ?

Intra-cellular Localization of Neurophysins and Hormones

It has long been known that the so-called neurosecretory material found in granules of paraventricular or supraoptic neurons gave specific staining reactions such as Gomori's chrom-alum-hematoxylin or Sloper's performic acid alcian blue, and could be identified with

cystine-rich proteins and possibly with neurophysins. More recent studies, particularly with immunofluorescence (Hope and Pickup, 1974) or immunoperoxidase (Sunde and Sokol, 1975) techniques have shown the presence of neurophysin-like material in cell bodies of magnocellular neurons in paraventricular and supraoptic nuclei. According to Hope and Pickup (1974), the immunofluorescent material follows a path from the hypothalamus to the posterior pituitary gland, which is identical to the path followed by the peptide hormones.

On the other hand neurophysins and hormones are found in the purified neurosecretory granules, and vasopressin seems associated to MSEL-neurophysin and oxytocin with VLDV-neurophysin (Hope and Pickup, 1974). Furthermore, in the mutant Brattleboro rat, deletion of the vasopressin gene is accompanied by the disappearance of a neurophysin as demonstrated by polyacrylamide gel electrophoresis (Pickering and Jones, 1978 ; Sunde and Sokol, 1975).

Immunological and Biochemical Identification of Precursors

Precursors biosynthesized by messenger-RNA. Preproneurophysins have been synthesized in cell-free systems through messenger-RNA. Precursors with molecular weights ranging from 17.000 to 23.000-25.000 daltons (Giudice and Chaiken, 1979 ; Lin et al., 1979 ; Schmale, Leipold and Richter, 1979), were identified usually by specific immunoreaction and sometimes by peptide mapping (Giudice and Chaiken, 1979).

Direct purification of common precursors. Preliminary research initiated by Sachs (Sachs et al., 1969) revealed in hypothalamus a macromolecular biosynthetic precursor of vasopressin and several data suggested that a neurophysin might be a part of this precursor. Later Gainer et al. (1977) and Brownstein et al. (1977) have shown by

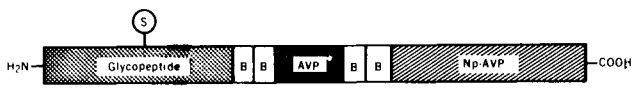


Fig. 8. Proposed structure of the common precursor. S, the sugar moiety of the glycopeptide ; B, basic amino acid residues ; AVP, arginine vasopressin ; NpAVP, the vasopressin-associated neurophysin (after Brownstein et al., 1980).

labeling experiments in rat supraoptic nuclei the presence of 20,000-dalton proteins which react with anti-rat neurophysin and which are rapidly cleaved into 12,000-dalton proteins and several smaller peptides. More recently a macromolecular common precursor (M_r 20,000, pI 6.1) reacting both with anti-vasopressin and anti-neurophysin has been purified. This precursor was subjected to limited proteolysis with trypsin which produced on one hand a M_r 10,000 protein identified as neurophysin on the basis of its pH-dependence affinity for vasopressin and its electrophoretic behaviour (pI 4.6-4.8), on the other hand peptides recognized by antibodies against vasopressin. The precursor would be constituted by an N-terminal glycopeptide, a central vasopressin and a C-terminal neurophysin (Fig. 8) (Brownstein et al., 1980).

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Relations Between the Structure and Biological Activity of Insulin*

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ABSTRACT

In order to understand the molecular basis of insulin action and the fate of the hormone in the organism, it is essential to obtain a complete picture of the relationships between primary structure, tertiary structure and biological properties. These studies require homogeneous analogues and derivatives. Strategies and tactics used in this Institute for the synthesis, semisynthesis and chemical modification of the native hormone are outlined. The procedures include total synthesis of A-chains, substitutions of amino and other functional groups, chain elongation, shortening of chains, replacement of amino acids and peptide fragments, and crosslinking. The effects of such modifications on biological activity in vitro and in vivo, on receptor binding, degradation, as well as on the structure of insulin in solution and in the crystal are described, and the results discussed on the basis of the threedimensional model of insulin. The paper summarizes results of cooperative structure-function studies carried out during the last 12 years.

KEYWORDS

Insulin, analogues, derivatives, chemical modification, semisynthesis, synthesis, biological properties, receptor binding, conformation, structure-activity relationships.

INTRODUCTION

Insulin is an anabolic hormone which plays a key role in the control of carbohydrate, fat and protein metabolism. The main target organs are the liver, muscle and adipose tissue. Best known is its effect on lowering the blood glucose concentration which appears to be mediated by interaction with specific receptors on the target cell membranes (For reviews, see Czech, 1976; Kahn, 1979; De Meyts, 1978). However, the exact mechanism of insulin action remains unknown.

**This paper is dedicated to Professor Dr. Helmut Zahn, Director of the Deutsches Wollforschungsinstitut, on the occasion of his 65th birthday (June 13, 1981).*

The hormone is indispensible in the treatment of juvenile and other forms of diabetes. In use for more than 50 years, beef and porc insulins can be considered as classical drugs. These have always been administered in their native form (Fig.1). Significant improvements of drug quality through modification of the original design have not yet been achieved.

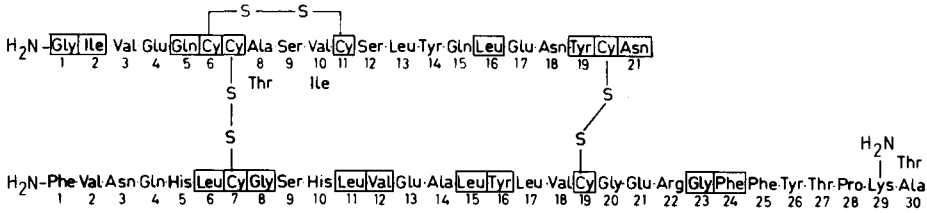

Fig. 1. Primary structure of bovine insulin (mol. weight 5734). Squares indicate invariant amino acids. About 30 insulins from different species have been sequenced so far (see Blundell and Humbel, 1980). Human insulin contains A8-threonine, A10-isoleucine and B30-threonine, porcine insulin A8-threonine and A10-isoleucine. N- and C-terminal extensions of the B-chain are found in fish insulins. The biosynthesis proceeds via the single-chain precursors preproinsulin and proinsulin. Presequences (24 amino acids) are attached to B1, connecting peptides (30-35 amino acids) link A1 to B30.

In view of the vital importance under physiological and pathological conditions, detailed investigations on relationships between primary structure, tertiary structure, and biological properties of insulin are of special interest. Since 1969, these can be based on the threedimensional model constructed from the atomic coordinates obtained from the X-ray analysis of insulin (Blundell and colleagues, 1972; Dodson and colleagues, 1979). Essential further prerequisites are the availability of homogeneous analogues and meaningful test systems. The first prerequisite has been a constant challenge to insulin chemists.

In our Institute, systematic studies to modify the primary structure of the native hormone began 12 years ago. They were based on the synthetic work which H. Zahn and coworkers had been persuing since the late fifties, and on model reactions with the intact molecule. Synthetic routes for the preparation of analogues were developed, and structure-function studies were started as joint ventures with other laboratories. The present paper summarizes approaches taken and results achieved, and outlines aspects of our current understanding of structure-activity relationships.

In view of the large number of insulin analogues and derivatives described in the literature (ca. 50 new compounds per year), reference to other work has to be limited. For a detailed picture of the present stand, the annual reviews on pancreatic hormones (Brandenburg and Saunders, 1979, and other volumes), and reviews by Geiger (1976), Dodson and colleagues (1979), as well as the Proceedings of the 2nd International Insulin Symposium 1979 (for complete reference, see Dodson and colleagues, 1980) should be consulted.

PREPARATION OF INSULIN ANALOGUES AND DERIVATIVES

There are four possibilities to obtain insulins with altered structure:
a) Isolation from different species

b) Modification of the native, usually bovine or porcine, hormone
c) Chemical synthesis
d) Synthesis applying gene techniques.

Many of the about 30 insulins isolated to date and also proinsulin, insulin-like growth factors (IGF I, II) and relaxin (see Blundell and Humbel, 1980) have been very valuable with respect to structure, function and evolution studies. Interpretations are often difficult since amino acid exchanges occur at various positions simultaneously. Human insulin has been obtained in E. coli via synthetic genes for A- and B-chains. This insulin has been thoroughly analyzed, and first results of biological and clinical studies have been reported (First International Symposium on Biosynthetic Human Insulin, Athens, Greece, Sept. 23, 1980. Proceedings to be published). Analogues have so far not been prepared. Routes b) and c) will be discussed below. In the preparation of these insulins, two aspects have to be taken into account, quality and quantity. Only homogeneous preparations allow for a clear interpretation of data, and only sufficient material makes a variety of investigations and correlations between biological and physical data possible.

Modification of the Native Hormone

The availability of native bovine and porcine insulins in a good state of purity, the stability of insulin over a considerable pH-range, its solubility in several organic solvents, and marked insensitivity towards denaturation facilitate this approach. Major problems reside in the fact that the molecule has a two-chain structure, that numerous functional groups are present and that many reagents exhibit only a limited specificity.

The primary structure of the hormone can be alterd by
a) the addition of monofunctional substituents to one or more reactive groups,
b) the cleavage of bonds in the main chain(s) or in, respectively between, side chains,
c) crosslinking, i.e. the introduction of new covalent bonds into one molecule or between molecules,
d) combinations of the procedures a - c, particularly of destructive (b) and constructive (a,c) methods.

Substitutions. Of all the functional groups (3 amino, 6 carboxyl, 4 aromatic and 4, resp. 5 aliphatic hydroxyl, 2 imidazole, 1 guanido), the amino groups are most important. In terms of peptide chemistry, insulin is a trifunctional amino component. Upon reaction with amino-specific reagents, 7 derivatives are to be expected: 3 mono-, 3 di- and one trisubstituted compound. As all reactions with the polyfunctional macromolecule are random, the primary reaction will give a complex mixture. Its composition will be determined by the reactivity of each amino group (pK-value, sterical factors), the nature of the substituting agent, and parameters as solvent, pH, etc.. As discussed in more detail by Geiger (1976) and by Brandenburg and colleagues (1981), amino group substitution is the basis for strategies and tactics aiming at sequential alterations of the structure.

As most substitutions of amino groups block protonation, analysis and fractionations are usually based on methods which differentiate according to charge. The preparation of acetylinsulins (Brandenburg and colleagues, 1972) illustrates these points. The reaction mixture, obtained by acylating insulin with p-nitrophenylacetate in dimethylsulphoxide containing an organic base, was first fractionated (Fig. 2) under conditions where charge differences are maximal. Subsequent separation of the resulting groups took advantage of the pK differences of α- and ε-amino groups. In this, and other reactions with activated esters of N-protected amino acids (see Friesen and others, 1977), 4 out of the 7 possible derivatives could be isolated.

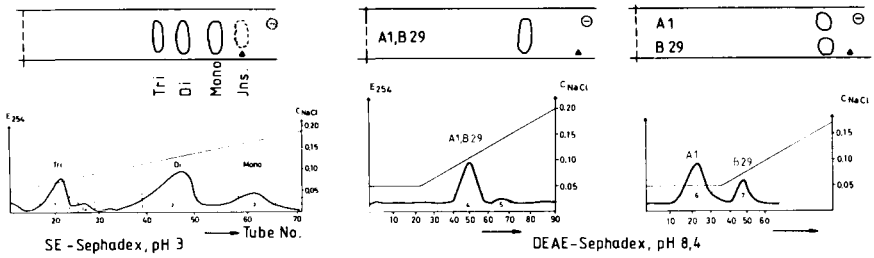

Fig. 2. Analysis of partially acetylated insulin and of purified derivatives by cellulose acetate electrophoresis at pH 2.4 (top). Fractionation according to substitution degree by ion exchange chromatography at pH 3 and subfractionation of di- and monoacetylinsulins according to substitution site at alkaline pH (bottom).

The reactivity of the amino groups towards p-nitrophenylesters has been found to be Gly > Lys >> Phe, while N-hydroxysuccinimide esters acylate preferentially Lys. Anhydrides in aqueous/organic mixtures at pH 5-7 react mainly with the N-terminals. The B1-amino group, usually of low reactivity, shows a marked selectivity for isothiocyanates. The order is Phe > Gly >> Lys. B1- and A1,B1-disubstituted ^{14}C-phenylthiocarbamoyl-insulins have been isolated (Brandenburg, 1969). These examples show that a limited number of derivatives can be obtaines by direct substitution of insulin. Specific substitutions at A1 could be achieved by acylating isolated A-chain, which is a monofunctional amino component, and subsequent combination with B-chain (Weinert and others, 1971, Brandenburg and colleagues, 1972).

Protecting groups. The application of protecting groups with different stability has opened the way to systematic and sequential alterations of insulin's structure. Four groups have been used: The acid-labile tert.butyloxycarbonyl (Boc) and citraconyl (Cit) residues, and the alkali-labile trifluoro acetyl (Tfa) and, in particular, the methylsulphoethyloxycarbonyl (Msc) residue. Fractionation of the primary reaction mixtures with citraconic anhydride and phenyltrifluoroacetate gave primarily A1,B1-di-, and A1-mono-protected insulins (Zahn and others, 1972, Friesen and others, 1977) while Boc-azide and Msc-N-hydroxysuccinimide ester yielded mainly A1,B29-diprotected derivatives (Geiger, 1976, Krail and others, 1975a, Weimann, 1977).

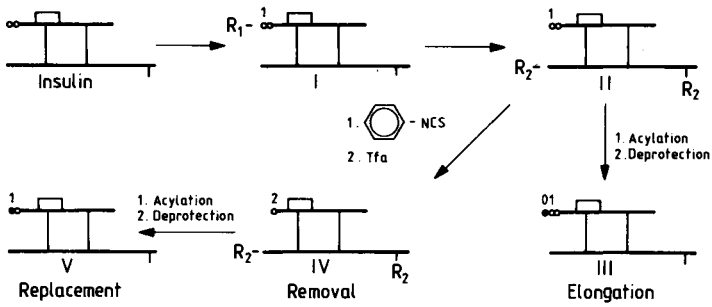

Fig. 3. Strategy for specific modifications and semisynthesis at the N-terminus of the A-chain. Temporary protection at A1 (R1), semi-permanent protection (R2) at B1 and B29, and deblocking at A1 gives II which is the key intermediate for all further steps (see text).

A1,B1-Cit_2-insulin was specifically acetylated at B29-lysine (Zahn and others, 1972). N-terminal extensions at the B-chain could be accomplished by reaction of A1,B29-Boc_2-insulin with a series of Boc-amino acid trichlorophenyl or N-hydroxy-succinimide esters (Gly, ε-Boc-Lys, Leu, γ-tert.butylester of Glu) and subsequent removal of all protecting groups (Krail and others, 1975a). The same approach was used to prepare B1-biotinyl-insulin (Hofmann and coworkers, 1977).

More complex protecting group tactics had to be developed for specific reactions at the N-terminus of the A-chain (Fig.3). The glycine amino group was first temporarily blocked with a Cit- or Tfa-residue. Then, B1 and B29 were protected with the Boc-group, and the A1 amino group deblocked to allow for acylation with a series of activated esters of Boc-amino acids, and also with guanyldimethylpyrazole (Friesen and others, 1977, 1978).

N-Terminal shortening of the peptide chains. Removal of N-terminal amino acids can be accomplished by an adaptation of the Edman degradation, well known from analytical protein chemistry. B1-mono- and A1,B1-di-phenylthiocarbamoylinsulins (see above) gave, upon treatment with trifluoroacetic acid, des-B1-Phe- and des-A1-Gly,B1-Phe-insulin (Brandenburg and Ooms, 1968, Brandenburg, 1969). In a second cycle, the latter analogue was further shortened by A2-Ile and B2-Val (Brandenburg and co-workers, 1971). After protection at A1 and B29 with the acid-stable Msc-group (Geiger, 1976), sequential degradation of the B-chain could be accomplished for 6 cycles (Weimann and Brandenburg, 1978). All six analogues were isolated in homo-geneous form after deprotection with alkali. Des-A1-Gly-insulin cannot be obtained directly from unprotected insulin. It was first prepared from des-Gly-A-chain through combination with B-chain (Brandenburg and coworkers, 1975a). Later, it became accessible following the elaborate protecting group tactics described in Fig. 3. Friesen and others (1978) used the Boc-group as R2, while Trindler (1980) applied the Msc-group.

Replacement of N-terminal amino acids. Edman degradation in conjunction with peptide synthesis has been used to study the functional role of the N-terminal phenylalanine and glycine. The first replacement of phenylalanine was accomplished using Boc protection at A1 and B29 (Krail and others, 1971). A1,B29-Msc_2-insulin (Geiger, 1976) was particularly suitable for semisynthesis at the N-terminus of the B-chain. After removal of Phe, a series of amino acids, including Trp, Met and D-Phe (Brandenburg and coworkers, 1978) and 3-iodo-desamino-tyrosine of high specific activity (Bahrami and others, 1980) have been introduced.

Replacement of A1-glycine was first accomplished by acylating des-Gly-A-chain with activated esters of several N-protected amino acids, (e.g. Leu, Pro, Val), deprotect-ion, and combination with B-chain (Krail and others, 1975b). Differential protect-ion of insulin (Fig. 3), using the Msc-group as R2, gave 10 analogues with α,β and γ amino acids, glycolic acid and other residues in position A1 (Trindler, 1980). Recently, A1-glycine has also been cleaved from B1-Msc-despentapeptide(B26-30)insulin, and been replaced by L- and D-Ala (Chu and colleagues, 1981).

Removal and replacement of C-terminal sequences. A series of analogues with shortened C-terminus of the B-chain has become accessible by enzymatic hydrolysis of insulin. Des-B30-Ala-insulin was obtained through the action of carboxypeptidase-A in ammonium hydrogencarbonate buffer (see Gattner and colleagues, 1981). Limited cleavage of insulin by pepsin at the bond B25-26 occurred when the hormone was ad-sorbed to a strongly acidic ion exchanger (Gattner, 1975). Des-pentapeptide(B26-30)-insulin was further degraded by carboxypeptidase-A, and counter current distribution yielded des-hexapeptide(B25-30) as well as des-heptapeptide(B24-30)insulin (Gattner

and colleagues, 1981). Further cleavage of the well-known des-octapeptide(B23-30)-insulin with carboxypeptidase-B produced des-nonapeptide(B22-30)insulin. Des-pentapeptide insulin has also been obtained from camel insulin (Danho and colleagues, 1975).

Semisynthesis concerning this region of the molecule had two main goals: Transformation of porcine to human insulin, i.e. the replacement of B30-Ala for threonine, and structure-function studies. The use of insulin as a carboxyl component poses major problems. Activation of one carboxyl group necessitates the protection of the amino groups and the five other carboxyl groups. Inspite of much work in this and other laboratories, chemical semisynthesis has so far been largely unsatisfactory (see Gattner and colleagues, 1981, and the other reviews). Enzyme-assisted peptide synthesis has now been shown to be an efficient way to overcome the problems. Based on the findings of Inouye, a variety of synthetic peptides and amino acid esters has been coupled to N-protected des-octapeptide insulin and to unprotected des-Ala-insulin by means of trypsin in mixtures of organic solvents and buffers (Gattner and colleagues, 1980,1981). Human insulin and several analogues were obtained in yields which depended strongly on the sequences and conditions employed, and ranged between 10 and 90%.

C-terminal modifications at the A-chain are limited, since only one amino acid is located outside the disulphide ring system. During saponification of insulin hexamethyl ester, cyclization occurred at A21, and [asparagine-imide]-insulin could be isolated (Gattner and Schmitt, 1977). [A21-isoasparagine]-insulin is the main product of further hydrolysis and could be separated from insulin.

Crosslinking. The introduction of new covalent bridges into macromolecules is a particularly interesting method of modification. The reaction of insulin with two types of amino-specific bifunctional reagents, m-phenylenediisothiocyanate and a series of activated esters of simple and more complex dicarboxylic acids has been studied in detail (see Brandenburg and coworkers, 1977 and Geiger, 1976, for review). Fractionation of reaction mixtures by gel filtration and subsequent electrophoresis or ion exchange chrcmatography yielded A1-B1 linked monomers and a B1-B1'-dimer with the former, and ca. 20 A1-B29-linked monomers as well as some A1-B1-isomers with the latter (Brandenburg and coworkers, 1971, 1973, 1975b, 1977). The length of intramolecular crosslinks was varied between 2 and 25 atoms (oxalic acid to ε,ε'adipoyl-bis-lysine).

For the preparation of homogeneous dimers, di-protected intermediates,mainly Msc-insulins (Schüttler and Brandenburg, 1979), were used. The three symmetrical dimers, linked with suberic acid between A1-A1', B1-B1' and B29-B29' could be prepared directly. For the three asymmetrical ones activated derivatives had to be prepared first. These were isolated and reacted with the second intermediate to give A1-B1'-, A1-B29'-and B1-B29'-suberoyl insulin dimers (Schüttler and Brandenburg, 1980).

Recently, a series of insulins with photo-activatable groups in positions A1,B1,B2 or B29 have also been prepared (for review, see Brandenburg and coworkers, 1981). These are being used for heterocrosslinking, particularly to receptors in isolated cells and membrans.

Modifications at cystine disulphide bonds. Partial thiol-disulphide exchange between insulin, thiols and disulphides of low molecular weight at pH 4.8 - 5 led to preferential formation of mixed disulphides with split A7-B7 bond. Derivatives in which the SH-groups of A7-cysteine and B7-cysteine were substituted with thioglycolic acid or glutathione were isolated in a state of about 75% homogeneity (Busse and Gattner, 1973b). A homogeneous A7,B7-S-sulfocysteine insulin could be obtained by

selective partial sulphitolysis of the A7-B7 bond (Busse and Gattner, 1973a). A synthetic modification of the A6-11 bond is described below.

Chemical Synthesis of Insulin Analogues

The synthesis of insulin requires the formation of 49 peptide bonds and 3 asymmetrical cystine bonds and is, despite considerable progress in peptide chemistry, still a formidable task (see reviews of Geiger, 1976; Büllesbach and Brandenburg, 1981). The elegant strategy of total synthesis via selective formation of each cystine bond has recently yielded several interesting analogues (Märki and colleagues, 1979) but has not yet been adopted by others. In our laboratory the preparation of analogues followed the "single chain strategy" designed for the first synthesis of insulin (Meienhofer and others, 1963). All A-chains were synthesized by fragment condensation in solution (Fig. 4).

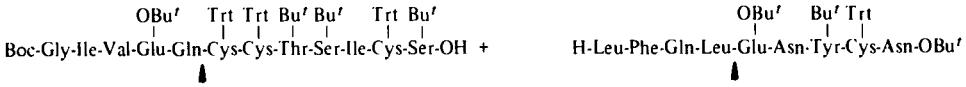

Fig. 4. Final fragments for the synthesis of [14-Phe]-A-chain. The sites of preceding couplings are indicated by arrows. From Danho and colleagues (1980).

Current protecting group tactics have been developed on the basis of detailed methodological studies and make use of trityl groups for thiol, and of tert.-butyl-ethers and esters for side-chain protection of hydroxyl- and carboxyl groups, respectively. Upon completion of the chains, all blocking groups are removed in one step by trifluoro acetic acid in the presence of thiophenol as a scavenger for trityl cations. Chains are always converted into the final form of S-sulphonates, which can efficiently be purified and analyzed. Typical yields of the purified chains, based on the "foreign" amino acid, are 7.6% for [Phe^{14}]-A-chain and 2.3% for [Phe^{19}]-A-chain. The insulin analogues are obtained through co-oxidation of the reduced A-chain with native B-chain and extensive purification of the resulting mixtures by ion exchange chromatography and gel filtration (Table 1).

TABLE 1 Analogues Obtained through Combination of Synthetic A-Chains and Natural B-Chains

Analogue	Molar ratio A:B	Yield mg	Yield %[1]	S-Protection	Reference
[Abu^{6}][2]	1:1.2	[3]	-	7,11:benzyl	Jost and coll., 1968
des-Gly^{A1}	1:1	5.9	4.2	6-7: SS 10-21: SS	Berndt and coll, 1975
[$Tyr(I)^{A19}$]	1:1	4[4]	9	trityl	Wieneke and coll., 1979
[Phe^{A14}]	1:1	16.8	6.2	trityl	Danho and coll., 1980b
[Phe^{A19}]	1:1	11	8	trityl	Danho and coll., 1980a

[1] Calculation based on purified A-chain. [2]Carba-insulin; cystathionine in positions A6,11, thus replacing the sulphur atom of 6-cysteine by CH_2. [3] Pure analogue not isolated. [4] H.-J. Wieneke, personal communication.

The notoriously low yields in such random reactions can be further influenced by the sequence of the analogous chain. Conversion of SH-group pairing from an intermolecular into an intramolecular reaction by crosslinking A- and B-chains between A1 and B29 gives yields up to 75% of the correctly formed insulin derivative (review: Geiger, 1976, Brandenburg and coworkers, 1977). This approach has, however, not yet been applied to synthetic chains.

EFFECT OF MODIFICATIONS ON THE PROPERTIES OF INSULIN

The systematic chemical studies described above have yielded several types of insulins: a) monomers with one, or two separated modified sites, b) monomers with intramolecular crosslinks, and c) insulin dimers. At the beginning, it could only be attempted to relate primary structure and biological activity (Brandenburg and Ooms, 1968, Jost and colleagues, 1968). Today, the aim of detailed investigations is to elucidate relationships between primary structure, threedimensional structure, receptor binding, biological action and degradation of the hormone. To illustrate some of these aspects, studies with about 50 insulins have been summarized in Table 2.

TABLE 2 Selected Insulins with Altered Primary Structure

	Analogue or Derivative[a]		Biological Properties[b] %i.vitro activity	%receptor binding	%i.vivo activity	Physical Properties[c] crystals	CD-spectrum	Reference[d]
	A-CHAIN							
1	A1-Acetyl		32-50	A 25 B 23	C 100	++	++	1,2,3,4 5
2	A1-Boc		17-19	A,B 15		++	++	3,5,6
3	A1-Arg		68 39-49	 B 107	D 57	+	++	7 2,5,6
4	A1-$(Arg)_3$		32-40	B 125				5,6
5	A1-Glu		22			++		6,8
6	[A1-Guanidoacetyl]		88	B 84		++		5,6,9
7	[A1-Acetyl]		15-30	B 18		++	+(10)++(11)	10,11
8	[A1-Carbamoyl]		12	B 12		++	+	11
9	[A1-Leu]		16	A 13	14	-	0	12
10	[A1-L-Pro]		2			-		12
11	[A1-D-Pro]		99	B 100		++	++	11
12	[A2-Guanido-Ile]		<3	B <1			0	11
13	des-A1-Gly		0.4-2	A 0.6	C10-26	-	0	9,11,13,14
14	[A14-Phe]	p	96				++	15,16
15	[A19-Phe]	p	23				+	17
16	[A19-Tyr(I)]	p	24					18
17	[A21-Asn-imide]		40		50		+	19,20
	B-CHAIN							
18	B1-Leu		75-77			+	++	20,21
19	B1-Gly		52-53			+	+	20,21
20	B1-Biotinyl		94	B 94				22
21	[B1-D-Phe]		61			+		20
22	[B1-Phe(I)]		65			+		23
23	[Desamino-Tyr(I)]		100			+		24
24	Des-B1-Phe		78-110	A 70; E	C 88	++	++	2,10,25,27
25	Des(B1-5)		9			-	++	16,28

Analogue or Derivative[a]	Biological Properties[b] %i.vitro activity	%receptor binding	%i.vivo activity	Physical Properties[c] crystals	CD-spectrum	Reference[d]
26 Des(B1-6)	9			-	+	16,28
27 [B24-Leu] h	12				o	16,29
28 [B25-Leu] h	3				++	16,29
29 Des(B26-30)	15-20	A 14; E	30	+	+	2,27,30
30 B29-Acetyl	75-90 F 92			+	++	1,4,31
31 Ftc	30			-		20
A- AND B-CHAIN						
32 Des-Gly,Phe	2	E	C 17 D 10	-	o	2,13,27,32
33 Des-Gly,Ile,Phe,Val	<0.2			-	o	10,13
34 Des-Gly,(B26-30)	0.7			-	o	33
35 A1,B1-Di-Msc	28			+		34
36 A1,B29-Di-acetyl	26-32 F 24	B25; E	C 100	+	++	1,2,27,35,36 4
37 A1,B29-Di-guanido	75-100	100				37
38 B1,B29-Di-Boc	45					9
39 A1,B1,B29-Hexa-N-methyl	77					38
40 [A7,B7-Cys(SO_3H)]			C 4-10 D 15		o	39,40
CROSSLINKED						
41 A1-B1-Pbc	1	B 0.5	C <1	-	o	2,10,36,40
42 A1-B1-Dodecoyl	0.6	B 0.2	C 4	-	+	2,36,40
43 A1-B29-Adipoyl	4-6	B 1.4	C 42	+	++	2,4,36,41
44 A1-B29-Diaminosuberoyl	4-6	B 6; E	C 63	++	++	2,4,36,42,43
45 A1-B29-Dodecoyl	7-13 F 5 (4)	A 11 B 2.3	C 86	+	+	2,35,40 4,36
46 B1-B1'-Pbc dimer	34-40	A 42; E B 46	C 16	-	+	2,40 36
47 B1-B1'-Suberoyl dimer	30 F 30	A 140		+	(16)	44
48 B1-B29'-Suberoyl dimer	76 F 76	A 184		-	(16)	44

a) Bovine insulins, except: p = porcine, h = human.[]= Replacement, Ftc = fluoresceine-thiocarbamoyl, Pbc = m-phenylenedithiocarbamoyl. b) Molar basis; insulin = 100%. Test systems: A=adipocytes, B=liver cell membrane, C=hypoglycaemia, D=mouse convulsion, E=cult.lymphocytes, F=antilipolysis. c)++ rhombohedral, + other crystal form; underlined: X-ray analysis. CD: ++ small, + moderate, O large changes.
d) Abbreviated references: 1.Brandenburg &c.1972, 2.Gliemann&Gammeltoft 1974, 3.Pullen &c. 1976, 4.Ellis &c. 1978, 5.Rösen &c. 1980, 6.Friesen &c. 1977, 7.Weinert &c. 1971, 8.Bedarkar &c. 1979, 9.Friesen &c. 1978, 10.Brandenburg &.c. 1971, 11.Trindler 1980, 12.Krail &c. 1975b, 13.Brandenburg &c. 1975a, 14.Berndt &c. 1975, 15.Danho &c. 1980b, 16.Wollmer,unpubl., 17.Danho &c. 1980a, 18.Wieneke &c. 1979, 19.Gattner&Schmitt 1977, 20.Brandenburg &c. 1978, 21.Krail &c. 1975a, 22.Hofmann &c. 1977, 23.Krail &c. 1971, 24.Bahrami &c. 1980, 25.Brandenburg 1969, 26.Wollmer &c. 1980, 27.De Meyts &c. 1978, 28.Weimann&Brandenburg 1978, 29.Gattner &c. 1980, 30.Gattner 1975, 31.Thomas &c. 1979, 32.Brandenburg&Ooms 1968, 33.Chu &c. 1981, 34.Schüttler&Brandenburg 1979, 35.Jones &c. 1976, 36.Freychet &c. 1974, 37.Friesen &c. 1979, 38.Uschkoreit &c. 1980, 39.Busse&Gattner 1973a, 40.Brandenburg &c. 1973a, 41.Brandenburg 1972, 42.Brandenburg &c. 1973b, 43.Dodson &c. 1980, 44.Willey &c. 1980.

Biological Properties

Activity determinations based on the hypoglycaemic effect are of course most directly related to the action of insulin in the organism. But since this effect is a product of different unknown factors, as resorption, transport, binding, action, degradation and possible counter-regulation(s), the interpretation of such data is difficult. A marked divergence of in vivo and in vitro potencies was already found in early experiments (Brandenburg and colleagues, 1972).

In vitro glucose utilization. Insulin stimulates glucose uptake and metabolism in isolated fat cells in a dose-dependent way (Fig. 5a). The standard assays for potency determinations are based on conversion of ^{3}H-labelled glucose into lipids, or on CO_2 production from ^{14}C-glucose (see Gliemann and Gammeltoft, 1974; Ellis and others, 1978). All modified insulin have been tested, and results can be summarized as follows (Table 2). Alterations at the N-terminus of the A-chain have variable effects, potencies range between 0.2 and 100%. Substitutions generally cause a moderate, removal of glycine almost a complete inactivation. The configuration of amino acids replacing Gly (compare 10 and 11) is critical (see Geiger and colleagues, 1980). Modifications of A19-Tyr or A21-Asn, but not of A14-Tyr, bring about a partial reduction of potency. The N-terminus of the B-chain is less sensitive to chemical changes. Potencies of insulins in which phenylalanine is substituted, removed, or replaced by a variety of other amino acids, range between 50 and 100%. Further shortening (see also Geiger, 1976) causes a gradual loss of activity which reaches a value of 10% after removal of 5 or 6 residues. Replacement of B25-Phe for Leu is the most effective of all B-chain modifications. Cleavage of the 5 C-terminal amino acids as well as substitution of the B29 amino group with a large residue lead to partial inactivation. The effect of blocking several amino groups appears to be additive, but substitutions of 2 or 3 amino groups without changing the positive charge do not reduce the activity significantly. A marked diminuition of potency is observed upon intramolecular crosslinking, particularly between the N-terminals, while insulin dimers retain a higher activity. All insulins gave a maximal stimulation at sufficiently high concentrations and thus appear to be full agonists.

Antilipolytic effect. Insulin inhibits adrenaline-induced lipolysis in adipocytes, and several derivatives (e.g. 36,45) have been tested to compare this property and lipogenesis (Ellis and others, 1978, Thomas and colleagues, 1979). The sensitivities for both responses were the same for the 4 compounds which have potencies of between 7 and 87%. These findings provide strong evidence that the different effects are mediated through the same set of receptors.

Receptor binding and in vitro activity. The affinity of insulin and analogues to receptors in different target tissues (see Fig. 5b) is usually determined by competition with radiolabelled insulin, e.g. in adipocytes (Gliemann and Gammeltoft, 1974), liver cell membranes (Freychet and colleagues, 1974) or cultured human lymphocytes (De Meyts and colleagues, 1978). In some cases ^{125}-I-labelled analogues have also been used (Willey and others, 1980, De Meyts and colleagues, 1980). In most cases, a good correlation between affinity and potency has been observed, and affinities to receptors in different tissues were similar. However, it has recently been found that several analogues with basic residues attached to A1, as the Arg-insulins (3,4), exhibit higher binding than activity (Rösen and colleagues, 1980). The dimers 47 and 48, but not 46, also show such a discrepancy. It appears that dimers can bind bivalently (Willey and colleagues, 1980; De Meyts and colleagues, 1980). Binding of A1-B29 crosslinked insulins (45 = 11%) and potency in adipocytes were well correlated (Gliemann and Gammeltoft, 1974), but the affinities of 11

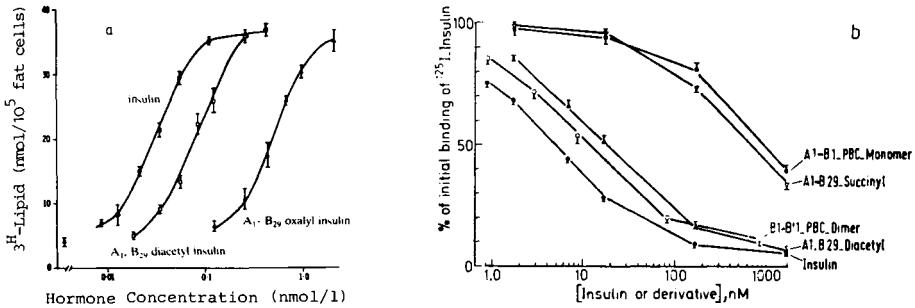

Fig. 5. a. Rat fat cell lipogenesis from 3-^{3}H-glucose (from Ellis and others,1978). b. Effect of native insulin and various insulin derivatives on binding of ^{125}I-insulin by liver plasma membranes (from Freychet & colleagues, 1974).

crosslinked derivatives to liver cell membranes ranged between 0.4 and 3.5% only. It has been concluded (see reviews) that in vitro potency reflects receptor binding, since in all cases, except hagfish insulin, similar figures have been obtained in both assays. Our studies generally support this view, but some A1-analogues, the dimers and possibly A1-B29 crosslinked derivatives are further interesting exceptions.

In vivo activity. About one third of the listed insulins have been tested either by determining blood sugar depression (in rats, rabbits or dogs) or by mouse convulsion assay. Potency figures from both assays are similar, and in some cases (e.g. 3,9, 17,29) are comparable to in vitro data. However, up to tenfold differences have been observed (3,32,36,42-45). By measuring plasma concentrations with specific immuno assays, Jones and colleagues (1976) demonstrated that degradation of A1-B29-crosslinked insulin (e.g. 45) was retarded. This led to rising plasma levels and consequently to an apparent higher potency.

Degradation. The mechanism of physiological inactivation of insulin is still unclear. Some studies have been carried out with modified insulins. The metabolic clearance rate of A1- as well as A1,B29-substituted derivatives (36), but not of B1 derivatives, was markedly reduced. A1-B29-crosslinked insulins (45) were particularly stable (Papachristodoulou and colleagues, 1977). Correlations between binding and degradation of several modified insulins (e.g. A1-B29-suberoyl-ins) by liver cells could be demonstrated (Terris and Steiner, 1975). On the other hand, the susceptibility of derivatives to cleavage by glutathione-insulin transhydrogenase (GIT), an intracellular enzyme, seemed to be unrelated to receptor binding (Papachristodoulou and colleagues, 1977). A1-B29-crosslinked insulins are markedly resistant towards attack by GIT, and also by proteolytic enzymes as chymotrypsin, trypsin and pepsin (Wang and colleagues, 1981).

Physical Properties

Crystallization and X-ray analysis. Precise information on the effects of modifications on the molecular architecture can only be obtained for the solid state, by X-ray analysis of crystals. Half-synthetic sheep insulin (Cutfield and others, 1979), A1-acetyl-, A1-Boc-insulin and also A1-thiazolidine-insulin (Pullen and others, 1976), A1-Glu-insulin (Bedarkar and others, 1979), des-B1-Phe-insulin (Wollmer and colleagues, 1980) and A1-B29-diaminosuberoyl-insulin (Dodson and colleagues, 1980) have been , or are still being, studied. Crystallization experiments are generally performed to obtain a first indication of gross structural integrity (see Table 2).

Circular dichroism. CD-Spectroscopy is presently the most important method to compare the structure of insulins in solution. The spectrum of insulin is complex, as it depends not only on the secondary/tertiary structure of the monomer, but also on the state of aggregation (see Wood and colleagues, 1975; Pullen and colleagues, 1976). While such overlap with phenomena of quarternary structure renders detailed interpretations very difficult, the dependence of spectra on hormone and zinc concentration is a valuable further parameter to assess molecular integrity. Fig. 6 illustrates the extent of spectral changes observed with insulin and an analogue with maximal spectral deviations. A gross classification of the modified insulins on the basis of reduction of ellipticities, shift of wave lengths, and reduced ability to aggregate indicates that for example analogues 7,11,14,24 and 44 are substantially unchanged, while 2,15,17,18,26,29 or 42 show moderate structural alterations. Pronounced effects of modification on conformation in solution have been noted for 12,13,40 and 41.

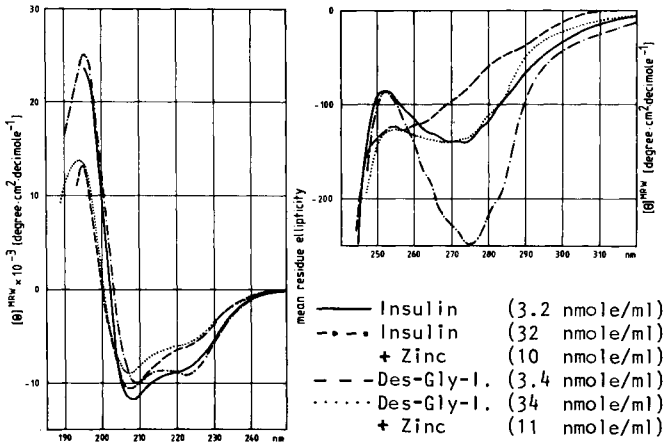

Fig. 6. CD-Spectra of porcine insulin and bovine des-A1-Gly-insulin in the far and near UV at pH 7.8 (Trindler; 1980, Wollmer, to be published)

Structure and Activity

A necessarily simplified synopsis shows that high potency and binding (>90%) is generally associated with minimal conformational changes, (see 6,11,14,23,24,30, 37), pronounced inactivation (<2% potency) with marked structural alterations (10, 12,13,32-34,41). Most insulins exhibit a partial diminuition of potency coinciding with moderate impairments of physical properties. Thus the threedimensional structure of insulin (Fig. 7) is rather sensitive to modifications at various sites, and losses of binding affinity and bioactivity are likely to be consequences of conformational changes. The crystal structure makes the effect of several modifications readily intelligible, e.g. the distortions induced by crosslinking remote groups (A1-B1, 41), and the structural compatibility of A1-B29 bridging (43-45). On the other hand, the N-terminus of the B-chain appears to form intra-monomer contacts in solution, but not in the 2-zinc crystal.

In view of insulin's deformability it is difficult to identify the residues which actually interact with the receptor. A putative binding region has been proposed (see Fig. 7) and parallels drawn between monomer-monomer and hormone-receptor association. Our experiments concern 3 of the 6 surface invariant amino acids and 3 of the 5 dimer forming residues. They show that the A1-carbonyl groups plays an

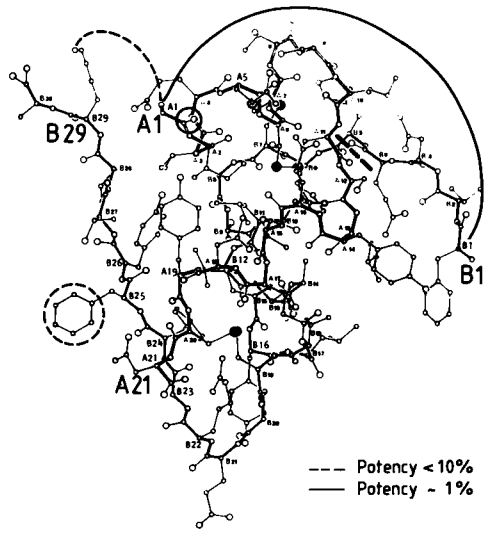

Fig. 7. Tertiary structure of insulin (monomer 1). Diagram from T. Blundell, with permission. 2-Zinc rhombohedral crystals contain hexamers in which 3 equivalent dimers are related by a rotational three-fold axis. Each dimer is formed through aggregation of 2 similar monomers. The dimer forming residues B12,16,24,25,26, and the surface residues A1,5,19,21, B22,23 constitute the putative receptor binding region (see Blundell and coll., 1972, Pullen and coll., 1976, Dodson and coll., 1979). Sites where modifications cause a marked inactivation (Table 2) are indicated.

important structural role; the A19-hydroxyl and A21-carboxyl groups as well as residues B24-Phe and B26-Tyr play limited structural roles. Generally, a correctly positioned positive charge in A1 is essential for full binding and potency, but not for an intact structure. Each of the other sites - A19, A21, B24, B26 - may be involved in binding, but it is not possible to assign the affinity and potency losses to perturbation of the defined residue or of a more remote site. None of these sites appear to be of singular importance; limited losses of activity would rather indicate a multipoint interaction. In contrast, the pronounced effect of Phe→Leu (28) replacement on potency, but not on the structure and association tendency of the monomer (Wollmer and colleagues, in preparation) points to a special role of the side-chain of B25. While conclusions about the receptor binding region on the basis of reversible interactions are indirect, insulins with photo-activatable groups at either position A1, B1/2, or B29 have been shown to form specific links to membrane receptors, directly implicating these sites as part of the potential binding region (see Brandenburg and coworkers, 1981).

In the considerations above, insulin has been treated as a static model, where deformation through modification hinders or abolishes the establishment of stereospecific contacts. Findings with crosslinked insulins would put more emphasis on dynamic aspects. According to X-ray analysis, CD-spectra and association studies, A1-B29-diaminosuberoyl insulin closely resembles insulin, but binding and potency are greatly reduced. It may well be that molecular flexibility is important, and that the crosslink hinders the molecule from adopting the optimal conformation for binding. Dodson and colleagues (1979) have extended this concept and suggest that such a loss of flexibility may generally contribute to potency losses seen after modification.

Chemical modification and semisynthesis on the one hand, and total synthesis on the other, are complementary techniques for the preparation of insulins with altered structure. With most of the modified insulins more information could be obtained than it is normally the case in structure-function studies with this hormone (see reviews). Biological and physical data have allowed a number of valuable clues to be drawn, but it is not yet possible to establish simple correlations between structural features and functional properties. Clearly, more modified insulins, further new types of derivatives, such as dimers or photo-activatable insulins, more refined physical methods and specific biological test systems are necessary to fully characterize this fascinating hormone. Once a complete picture is obtained, this may hopefully be used for the design of molecules with improved therapeutic properties.

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