ANNUAL REPORTS IN MEDICINAL CHEMISTRY Volume 16

Sponsored by the Division of Medicinal Chemistry of the American Chemical Society

Editor-in-Chief: ***HANS-JÜRGEN HESS***

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Volume 16

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SECTION EDITORS

JOHN McDERMED · WILLIAM COMER · LESLIE WERBEL
DENIS BAILEY · EUGENE CORDES · RICHARD ALLEN

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PREFACE

In addition to the traditional chapters providing a general update of research progress related to the major therapeutic categories and special topics reviewed periodically, Volume 16 contains several chapters that discuss in greater depth recent scientific advances and trends that seem to offer particular promise for new drug development. Thus, included in this volume are chapters on benzodiazepine receptors, renal vasodilators, calcium antagonists, and arachidonate lipoxygenase. A chapter on parasite biochemistry is complementing the traditional chapter on antiparasitic agents and, for the first time, we have included a comprehensive review of veterinary drugs. Although we were not successful in including, as planned, an overview chapter on recombinant DNA research, specific applications of this technology are mentioned, for example, in the chapters on diabetes and human interferons, while advances in sequencing and synthesis of nucleic acids are discussed in a separate chapter. Also timely is the chapter on monoclonal antibodies, another area of wide current interest and effort.

A cumulative keyword index of chapter titles has been added this year to complement the cumulative titles index organized by sections.

To all the individuals who contributed their time and effort to make this volume possible, particularly to Ms. Mary Heinold whose help was invaluable in assembling the manuscript and preparing it for photoreproduction, we extend our sincere gratitude and appreciation.

Groton, Connecticut
May 1981

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Section I - CNS Agents

Editor: John McDermed, Burroughs Wellcome Company,
Research Triangle Park, N.C. 27709

Chapter 1. Antidepressants

Carl Kaiser and William E. Bondinell
Smith Kline & French Laboratories, Philadelphia, PA 19101

Introduction - Amoxapine became the eighth tricyclic antidepressant (TCA) to be marketed in the United States. Several other antidepressants, e.g., quinupramine, amineptine, trazodone, etoperidone and clomipramine, have been introduced abroad, and at least 30 other antidepressants are in various stages of clinical development. In addition, a great many clinical, pharmacological, pharmacokinetic, metabolic and, perhaps most importantly, mechanistic studies of antidepressants have appeared. A prime motivation for such intense research is the development of more efficacious, less toxic and more rapidly acting antidepressants. The side effects of existing agents, particularly cardiotoxicity,[1,2] anticholinergic actions,[3] neuroendocrine dysfunction,[4] akinesia,[5] etc., have been the subject of comprehensive reviews.[6-8] In addition, several general reviews on depression[9,10] and antidepressants,[11-14] as well as their clinical,[15,16] pharmacokinetic[17-19] and pharmacological aspects,[20] have appeared. Newer methods of diagnosis and selective treatment of specific depressive disorders have also been reviewed.[21] Recent advances in predictive test procedures and in an understanding of the mechanism of action provide a basis for optimism that novel efficacious antidepressants may be forthcoming.

Tricyclic Compounds with Antidepressant Activity - Few new TCAs were described during the past year. Several series, e.g., 1-3, bear a piperazinyl side chain. The thienobenzoxazepine 1 was 0.4 times as potent as imipramine as an antagonist of tetrabenazine-induced depression in mice; it also caused neuroleptic effects, as it afforded protection against amphetamine-induced lethality in grouped mice. The related thiazepine 2 was about 3 times more potent than imipramine in the antitetrabenazine test but did not protect against amphetamine lethality.[22] The pyrrolobenzodiazepine 3, one of a series, had dual activity. It was 1.5 times more potent than imipramine in a tetrabenazine antagonist test in mice and had an ED_{50} = 4.7 mg/kg, p.o., in the amphetamine lethality procedure.[23] In a series of alkylaminoethylthiodibenzoxepins, 4 was 4 times more potent, and its 10,11-dihydro derivative was 1.6 times more potent, than imipramine in preventing tetrabenazine-induced ptosis in mice.[24] Other new TCAs are 5-7. Aminoacyl (e.g. 5), and aminoalkyl (e.g. 6) derivatives of indolobenzodiazepines potently inhibit tetrabenazine-induced ptosis in mice (2.2 and 3 times amitriptyline, respectively),[25] whereas the octahydrodibenzazepinonaphthyridine isomers 7a and 7b, rigid congeners of imipramine, have TCA-like activity in blocking norepinephrine (NE) and dopamine (DA) uptake and in binding studies with rat brain homogenate. The trans isomer 7a, a potential

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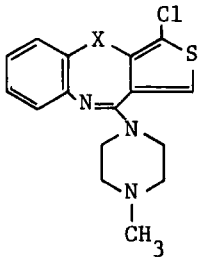
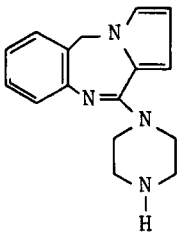
antidepressant, is a fairly potent and selective inhibitor of NE uptake, with some central anticholinergic and anti-DA but little α-adrenergic blocking activity. Binding studies suggest possible neuroleptic properties for the cis isomer 7b.[26]



1, X=O 2, X=S



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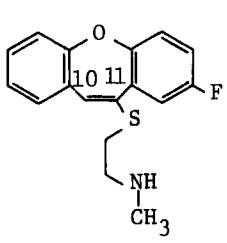

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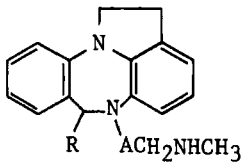
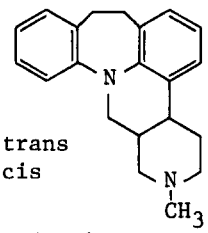
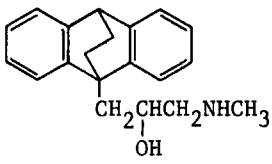


5, R=CH_3; A=C=O

6, R=H; A=$(CH_2)_2$



7a, trans
7b, cis



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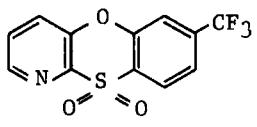
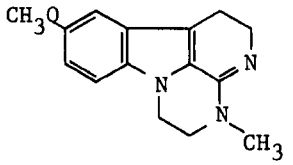


9

Another imipramine derivative, 3-cyanoimipramine (Ro 11-2465), blocks DA receptors[27] and has two high affinity serotonin (5-HT) binding sites in rat brain. It is uncertain whether these are two receptors for 5-HT uptake or one site that is related to uptake and one that is not.[28] NE-uptake inhibitory activity of the clinically effective hydroxymaprotiline (8, C 49-802 BDA),[29] resides mainly in the S-enantiomer.[30] The azaphenoxathiin dioxide 9, which lacks a TCA-like side chain, is twice as potent as imipramine in reversing tetrabenazine-induced ptosis in mice.[31] The molecular and conformational aspects of TCAs were compiled and reviewed.[32]

Non-tricyclic Compounds with Antidepressant Activity - Many studies and reviews have focused on previously reported non-TCAs. One of these summarizes the actions of the tetracyclic indole pirlindole (pyrazidol), which has TCA-like activity in animals[33] and is a clinically effective antidepressant.[34] A somewhat related compound, inkasan (10), has characteristic antidepressant properties and has been approved for clinical use in Russia.[35] Another tetracyclic indole, the prototype of a series, with reserpine antagonist activity is 11.[36] Other indoles with antidepressant actions are 12, which is twice as potent as imipramine in an antitetrabenazine-induced ptosis test in mice;[37] and binodaline (13), which has similar activity in rats and is being studied clinically.[38] RU-24969 (14) inhibits rat brain 5-HT binding;[39] and Wy 25093 (15) is a potent and selective inhibitor of 5-HT uptake into cerebral cortex slices from rats and monkeys.[40] Indalpine,[11] was selected from a series for clinical study because of its brain 5-HT uptake inhibiting activity.[41]



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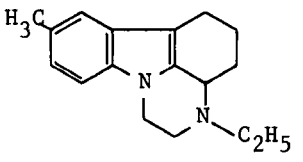

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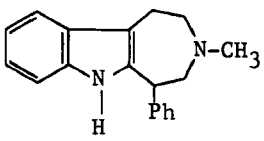

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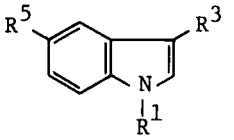
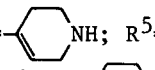
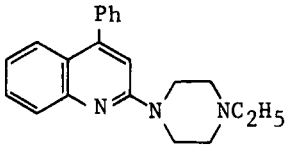


13, R^1=N(CH$_3$)(CH$_2$)$_2$N(CH$_3$)$_2$; R^3=Ph; R^5=H

14, R^1=H; R^3=  ; R^5=CH$_3$

15, R^1=R^5=H; R^3=CH$_2$N -NHCONHCOPh

1-(3-Dimethylaminopropyl)-5-methyl-3-phenylindazole (FS-32)[11] and its monomethyl homolog (FS-97) are comparable to amitriptyline as inhibitors of rat brain synaptosomal uptake of DA.[42] Both preferentially inhibit type B MAO,[43] but FS-97 is slightly more potent than its tertiary amine counterpart in a mouse antireserpine test.[44] 2-(4-Ethylpiperazinyl)-4-phenylquinoline (16, AD-1308), the most potent of a series of 2-aminoquinolines,[45] was more effective than imipramine in a variety of behavioral models and other tests for antidepressant activity.[46] Antidepressant activity was also suggested for 17, an inhibitor of prochlorperazine-induced catalepsy in mice,[47] and for 18 (K-8409), which is about one-half as potent as imipramine in antagonizing pentylenetetrazole-induced convulsions in mice.[48] Two amidines with antidepressant-like activity are naphthalene-2-acetamidine (19, DL-588), a reserpine antagonist in mice and rats,[49] and nitrafudam (20), a more potent tetrabenazine antagonist than imipramine in the same species.[50] The imidazolidine-2,4-dione 21 also antagonizes tetrabenazine-induced ptosis in mice but does not inhibit biogenic amine uptake into rat brain synaptosomes.[51]



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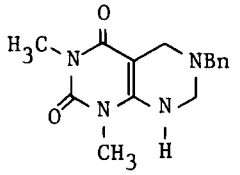

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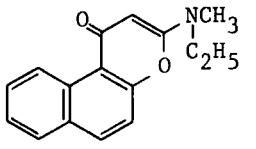

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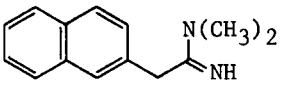

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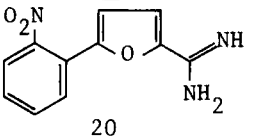

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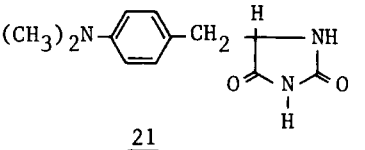
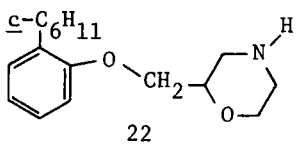


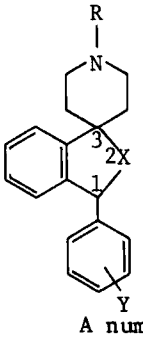
21

Antidepressant activity is reported for many aryloxyalkylamines. A series of cis- and trans-3-aryl-4-aryloxypiperidines and related sulfides has antitetrabenazine effects in mice. One of the more potent members of this series is trans-4-(4-bromophenoxy)-3-phenyl-1-methylpiperidine.[52] Among a series of benzhydryloxyethylpiperazines, potent inhibition of rat brain synaptosomal uptake of DA was noted for (4-FC$_6$H$_4$)$_2$CHO(CH$_2$)$_2$-c-N(CH$_2$CH$_2$)$_2$N(CH$_2$)$_3$Ph, which had an IC$_{50}$ = 2.0 nM.[53] Several 2-substituted phenoxybutylamines also have antidepressant actions. For example, 2-BnC$_6$H$_4$O(CH$_2$)$_4$NHCH$_3$ (MCI-2016) is more potent than imipramine in reversing reserpine-induced hypothermia in mice.[54,55] Some phenoxyamidoximes and imidazolines, e.g., 2-(c-C$_6$H$_{11}$)C$_6$H$_4$OCH(CH$_3$)C(NH$_2$)=NOH (MG-18415), were more potent than imipramine as antagonists of reserpine-induced ptosis in mice, but they were less effective in counteracting hypothermia.[56] Marked antidepressant-like pharmacology was also noted for the related aryloxymorpholine (22)[57] and the aryloxypropanolamine centpropazine, 4-(H$_5$C$_2$CO)C$_6$H$_4$OCH$_2$CH(OH)CH$_2$-c-N(CH$_2$CH$_2$)$_2$-NPh, which blocks reserpine-induced depression in mice, potentiates amphetamine-induced effects in rats and has clinical activity similar to that of imipramine.[58]



22

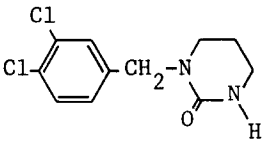
Spiro phthalane- and thiophthalane-piperidines have been studied extensively. HRP-197 (23) potentiates NE, DA and 5-HT systems and has minimal anticholinergic activity.[59] A thio analog (24) is 2 to 3 times more potent than imipramine in a mouse antitetrabenazine test.[60] These compounds are weak inhibitors of MAO, but are potent and selective inhibitors of 5-HT uptake.[61] Structure-activity relationship studies on enantiomers of 1-(3-aminopropyl)-3,3-dimethylphthalanes and related compounds as inhibitors of NE uptake indicate that the most active compounds have phenyl and aminopropyl substituents in position 1 oriented so that the structures are superimposable on a model of desipramine (DMI). Apparently the methyl groups in position 3 contribute to potency via ancillary binding.[62]



23, X=O; R=H; Y=4-CH_3

24, X=S; R=CH_3; Y=2-Cl

A number of newer antidepressants have concomitant antianxiety activity. For example, 25 inhibits NE uptake and combines tetrabenazine and pentylenetetrazole antagonist actions in mice.[63] Pyrazinobenzodiazepines, e.g. 26, weakly inhibit NE and 5-HT uptake into rat brain synaptosomes and have an antianxiety profile.[64] Both antianxiety and weak antidepressant actions are noted in the pharmacology of triazolobenzodiazepines, e.g. 27[65] and 28.[66] Alprazolam (29) was as effective as imipramine in a clinical study with depressed patients.[67] Clinical effectiveness,[68] combined with an antidepressant profile,[69] is described for zometapine (30). The 1,3-benzodiazepine HRP 453 (31) is a potent inhibitor of tetrabenazine-induced ptosis.[70]



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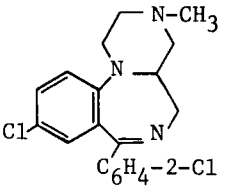

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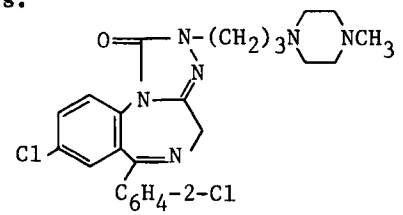

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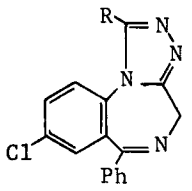
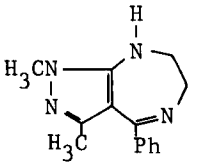


28, R=$CH_2N(CH_3)_2$

29, R=CH_3



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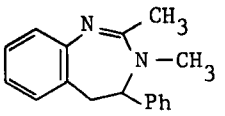

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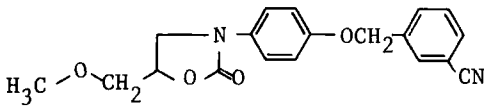
Monoamine Oxidase and Inhibitors (MAOIs) - MAO and its inhibitors were the subject of an extensive review.[71] Clinical studies have described the efficacy of both type A[72] and type B[73,74] MAOIs in depression. Clinical studies describe the efficacy of combined TCA-MAOI treatment.[75] A definitive double-blind controlled clinical comparison of an MAOI (phenelzine) and a TCA (amitriptyline) suggests therapeutic indications for each.[76] Two new type A selective MAOIs, MD 780515 (32)[77] and 6-methoxy-1,2,3,4-tetrahydro-β-carboline (33),[78] were described, and the properties of benzylhydrazine, a selective type B MAOI, were reviewed.[79] The effect of tranylcypromine enantiomers on neurotransmission and

behavior was reviewed. The d-isomer influences mainly 5-HT neurotransmission, whereas the l-isomer affects primarily catecholaminergic neurotransmission.[80] In rat brain, intrasynaptosomal mitochondria have about a 3.4 fold higher MAO A:B ratio than extrasynaptosomal mitochondria. Because DA deamination proceeds by specific uptake into these particles, type A MAO is especially important in DA neuron terminals; type B may function as an inactivator of other amines that enter the brain.[81] Mechanistic studies indicate that mitochondrial amine oxidation involves two one-election transfers from the substrate to the flavin.[82]



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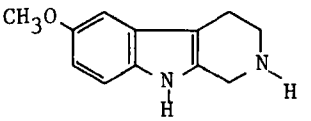

33

Miscellaneous Antidepressants and Therapy - Amino acid precursors of neurotransmitter amines and peptides continue to receive attention as potential antidepressants; however, their utility is controversial. Clinical studies and reviews suggest some beneficial effects with phenylalanine,[83,84] tryptophan,[85] 5-hydroxytryptophan,[86] tyrosine,[87] triiodothyronine,[88] S-adenosylmethionine[89] and thyrotropin-releasing hormone (TRH)[90] in the treatment of depression. Clinical studies continue to verify the utility of lithium (Li) in the treatment and prophylaxis of unipolar[91] and bipolar[92] depression, as well as in aggressive mentally subnormal patients.[93] Nevertheless, the mechanism of Li's antidepressant action remains unclear. Recent evidence indicates Li slows transport of choline to and from the brain,[94] a finding perhaps relating to the hypothesis that central acetylcholine is less available during depression.[95] The observation that chronic treatment with therapeutic concentrations of Li inhibits NE-induced c-AMP accumulation[96] may be of mechanistic significance. Other observations, e.g. extrapyramidal effects after Li therapy[97] and increased DA metabolites, may implicate a dopaminergic mechanism. The notation of decreased binding of 5-HT and GABA after chronic, but not acute, Li administration may have mechanistic implications.[97a] Miscellaneous agents reportedly of benefit in the treatment of depression include β-adrenergic receptor agonists,[98] such as salbutamol[99] and clenbuterol,[100] benzodiazepine anxiolytics,[101] antipsychotic drugs such as sulpiride,[102] theophylline,[103] and folic[104] and ascorbic acids.[105] In addition, several compounds have unique central stimulant actions. For example, 4-$FC_6H_4(Ph)CHO(CH_2)_2$-c-$N(CH_2CH_2)_2N(CH_2)_2COOH$ was the most potent member in a series; it increased exploratory behavior in mice without causing amphetamine-like central stimulation, and is being studied clinically for "attention mobilizing ability."[106]

Electroconvulsive therapy (ECT) remains an effective means of treating depression,[107] although side effects, especially impaired memory, are frequent. The antidepressant efficacy of ECT may result from increased release and greater penetration into the brain of hypothalamic peptides with behavioral effects.[108] Sleep deprivation is also frequently cited as a treatment for depression.[109]

Screening Methods - MAO inhibition is generally measured in vitro using brain or liver homogenates. TCAs are frequently identified by their ability to block uptake of NE, DA or 5-HT into brain preparations in vitro. Pharmacologic procedures most frequently used are ability to prevent or reverse effects, e.g., ptosis, depression, hypothermia, etc.,

induced by reserpine, tetrabenazine and other short-acting depletors of monoamine stores. As many of the newer non-classical antidepressants are ineffective in these traditional tests, new procedures capable of identifying antidepressants with differing mechanisms are being sought. To date, most successful procedures of this kind involve created animal models of depression, e.g., separation despair and behavioral helplessness.[110] One of the most predictive tests involves effectiveness in shortening the "despair" time of mice or rats placed in individual containers of water.[111] This procedure is predictive for non-classical antidepressants such as Li and mianserin.[112] The neurochemical basis of this test[113] may involve blockade of presynaptic α-adrenergic receptors[114] or reduction of steady-state 5-HT levels.[115] Antagonism of clonidine (an α_2-adrenergic receptor agonist) induced sedation[116] and enhanced clonidine-induced aggression are also caused by chronic antidepressant administration,[117] as is antidepressant potentiation of TRH-induced hyperthermia.[118] Many antidepressants were also effective in antagonizing hyperactivity induced in rats by long-term isolation (social despair).[119] Another behavioral model measures the effect of antidepressants under a differential reinforcement of low rates schedule in rats. TCAs decrease response and increase reinforcement rate.[120] Olfactory-bulb ablation of rats causes an acquisition deficit in a passive avoidance test[121] and an inability of the animals to respond to low temperatures. The abnormal response to cold was reversed by various antidepressants.[122] The deficit in passive avoidance acquisition was blocked by a variety of antidepressants, apparently by a mechanism involving blockade of 5-HT uptake; it was also blocked by several GABAergic drugs.[123]

Other new pharmacological tests for antidepressants involve attenuation of amphetamine-induced anorexia in rats,[124] blockade of apomorphine-induced hypothermia in mice[125] and reversal of amphetamine-induced depression of self-stimulation rates in rats.[126]

Mechanism(s) of Action - Research on the mechanism(s) of action of antidepressants has focused on changes in receptor sensitivities that occur with chronic, but not acute, administration of antidepressants and on high-affinity binding of TCAs. Recent progress and the roles of α_2- and β-adrenergic receptors and of NE receptor-coupled adenylate cyclase in depression and in the mechanism of action of antidepressants have been reviewed.[127-132] Subsensitivity of α_2-adrenergic receptors occurred in rat brain following chronic administration of DMI and in peripheral tissues following chronic TCAs and iprindole.[133-135] The decrease in β-adrenergic receptor density in rat cortex seen after chronic administration of DMI was accelerated by co-administration of yohimbine or phenoxybenzamine;[136,137] yohimbine also accelerated the increase in α_2-adrenergic receptor density seen with chronic DMI.[136] Ligand binding and bioassays were used to determine the affinity of antidepressants for α_1- and α_2-adrenergic receptors in rat cortex, calf frontal cortex and in peripheral tissues.[138-142]

Chronic administration of TCAs, MAOIs, iprindole and bupropion, but not of mianserin or other psychotropic drugs, decrease β-adrenergic receptor binding in rat cortex.[143] Mianserin and zimelidine reduce the NE-stimulated c-AMP response, but not the density of β-adrenergic receptors, in rat cortex following chronic administration.[144] Circadian rhythms of α- and β-adrenergic receptors in rat brain were altered by long-term administration of imipramine.[145] Chronic administration of TCAs, MAOIs or iprindole caused a decrease in 5-HT and β-adrenergic receptor densities in rat cortex.[138,146,147] Electrophysiological studies showed enhanced sensi-

tivity to postsynaptic, but not to autoreceptor-mediated, effects of 5-HT after chronic TCAs.[148-150] Behavioral studies showed enhanced responses to 5-HT following chronic administration of antidepressants.[151] DA autoreceptor subsensitivity to apomorphine was observed with chronic TCAs and iprindole and with electroconvulsive shock (ECS);[152,153] after longer administration of antidepressants, electrophysiological studies showed some neurons were excited by apomorphine.[153] Enhancement of 5-HT- and DA-mediated behavior in rats by repeated ECS requires intact brain NE systems.[154] α_1-Adrenergic and muscarinic receptor binding were unaffected or increased by long-term administration of antidepressants; DA receptor binding was not affected.[138,146,155,156] High-affinity [^{3}H]-imipramine binding has been demonstrated in human brain and platelets and in rat brain; binding in rat brain, which may be associated with the 5-HT reuptake site, was reduced by chronic administration of DMI and by REM sleep deprivation.[157,158] High-affinity [^{3}H]-mianserin binding was noted in rat cortex and calf caudate.[159,160]

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Chapter 2. Antipsychotic Agents and Dopamine Agonists

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While research investigations have continued to evaluate critically the role of central hyperdopaminergic processes in the complex pathogenesis of the collection of diseases known as schizophrenia, several alternate theories have also been advanced or reviewed during the past year.[1] These theories include a unifying hypothesis of schizophrenia based on hyperformaldehydism,[2] a neuroanatomical theory concerning the etiology of these diseases,[3] an hypothesis dealing with opiate receptors and endogenous neuropeptides,[4] and a proposal based on dopamine (DA) receptor supersensitivity resulting from a DA deficiency.[5] With respect to the DA theory of schizophrenia, arguments have recently been presented that increased dopaminergic transmission produces the positive symptomatology of schizophrenia (Type I syndrome, equivalent to an acute state of the disease), while other factors, such as structural changes in the brain, produce the negative symptomatology of schizophrenia (Type II syndrome, equivalent to the defect state of chronic schizophrenia).[6,7] The underlying pathological process in the Type I syndrome is posited as an increase in DA receptors, and, thus, the potential for clinical response to neuroleptic drugs is good. The Type II syndrome, on the other hand, differentiates a sub-group of schizophrenics that would show a less than favorable response to conventional antipsychotic therapy. Dopamine receptor supersensitivity, resulting from chronic DA receptor blockade by neuroleptic drugs, has been implicated in the pathophysiology of tardive dyskinesia. Recent review articles have addressed this consequence of antipsychotic therapy, as well as neuroleptic-induced parkinsonism.[8-10] Research in Parkinson's disease, wherein degeneration of DA neurons in the nigrostriatal pathway results in a pathological DA deficiency, continues to center on the therapeutic use of direct acting DA agonists. Such agonists are also being studied in schizophrenic patients as alternatives to conventional neuroleptic drugs.[11] Moreover, a selective presynaptic DA receptor agonist may have the beneficial effect of a reduced potential for extrapyramidal side effects and tardive dyskinesia. Recent developments in the fields of DA receptors, antagonists and agonists are considered in this review.

Dopamine Receptors - The D-1 (linked to adenylate cyclase) and D-2 (not linked to adenylate cyclase) DA receptor classification of Kebabian and Calne[12] remains the most widely accepted of the DA receptor concepts, and the nature of these receptors has been further characterized during the past year. For example, new data have been presented that ^{3}H-*cis*-flupenthixol is a selective ligand for the D-1 receptor,[13] while ^{3}H-spiroperidol has again been shown to block both D-1 and D-2 receptors.[14,15] The binding of ^{3}H-sulpiride, and other benzamide antipsychotic agents that are selective antagonists at the D-2 receptor, has been found to be dependent on the presence of sodium ions.[16,17] This cation is not required, however, for the binding of ^{3}H-spiroperidol or other classical neuroleptics.[17] Guanosine triphosphate (GTP) has previously been reported to modulate DA agonist binding solely at D-1 receptors.[18] Recent studies now suggest that GTP may also regulate agonist binding at some D-2 receptors.[14] Also, GTP-sensitive and insensitive agonist binding sites have been detected in calf retina,[19] a tissue which, until recently,[20,21] was thought to contain a homogeneous population of D-1 receptors.[22]

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Operational definitions of new categories of the DA receptor continue to be generated from receptor binding studies. For example, a D3 DA receptor has been proposed by Seeman and his colleagues,[23] and phenoxybenzamine has been used to distinguish between multiple classes of DA receptors.[24] Dopamine agonist and antagonist binding sites have been thermally differentiated.[25-27] A novel method of distinguishing pre- from postsynaptic DA receptor binding sites has been proposed by Sokoloff et al. who reported two classes of apomorphine (APO; 5a) binding sites in rat striatal tissue.[28] At one set of binding sites, designated class I, ^{3}H-APO is displaced by low (nM) concentrations of domperidone. The number of class I sites is greatly reduced when cell bodies within the striatum are destroyed with kainic acid. This implies that class I sites reside on intrinsic striatal neurones. At the other set of binding sites, designated class II, ^{3}H-APO is displaced by higher concentrations of domperidone. Moreover, the number of these sites is not reduced following the intrastriatal injection of kainic acid which destroys cell bodies while sparing axons of passage and nerve terminals. This implies that class II sites exist presynaptically on the nerve terminal.

Not all classifications of the DA receptor have been based solely on data from receptor binding studies. Dopamine receptors have been categorized also as either excitatory (DAe) or inhibitory (DAi) on the basis of behavioral, electrophysiological, and anatomical criteria.[29]

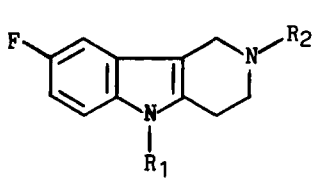
Recent reports have again confirmed that chronic treatment of rats with antipsychotic drugs leads to an increased number of DA binding sites,[30] and that such an increase may mediate the behavioral supersensitivity to APO seen in the rat during withdrawal or termination of drug treatment. This supersensitive response has been used as a model of tardive dyskinesia. Interestingly, estradiol and certain synthetic estrogens such as moxestrol share common pharmacological properties with neuroleptic drugs, especially in the above animal model. Thus, acute administration of each blocks the postsynaptic DA-like action of d-amphetamine and APO.[31-33] Chronic administration of each, however, causes an increase in ^{3}H-spiroperidol binding sites in striatum.[34,35] The pituitary gland is intimately involved in the antidopaminergic activities of these estrogen-like compounds since hypophysectomized animals no longer show drug related behavioral and biochemical effects.[32,35] Withdrawal from chronic estrogen treatment also causes behavioral supersensitivity to APO in 6-OH-DA lesioned rats.[33,34,36] Of possible therapeutic significance is the report that certain estrogens can replace neuroleptic agents in blocking the supersensitive response to APO in this animal model.[31]

Calmodulin is a regulatory protein which reduces DA activation of adenylate cyclase. This protein has been implicated in the development of dopaminergic supersensitivity following long-term administration of neuroleptics since such treatment inhibits the release of calmodulin.[37] Thus, membrane bound calmodulin is increased in brain tissue of animals given chronic neuroleptic treatment.[38] No significant difference has been found, however, in calmodulin content between post-mortem brains of schizophrenic and normal patients.[39]

During the past year, autoreceptors for DA, which are thought to mediate sedation,[40] were found to be rendered subsensitive following treatment with tricyclic antidepressant drugs.[41] This finding may partially explain the efficacy of these compounds in alleviating depression.[42,43] Chronic neuroleptic treatment, on the other hand, renders these DA autoreceptors supersensitive,[44] an effect that is blocked by the concurrent administration of lithium.[45]

Antipsychotic Agents - A full account of the structure-activity relationships of neuroleptic 5-aryltetrahydro-γ-carboline derivatives has now appeared.[46] This class of compounds is distinguished not only by potent activity in antagonizing

d-amphetamine-induced stereotypy in rats, but also by duration of neuroleptic activity. The racemate 1 (flutroline, CP-36,584), the premier member of this series, has now reached clinical evaluation as an antipsychotic agent. Further, Pfizer scientists have extended their studies to include the two racemic diastereoisomers and the two pairs of enantiomers of 2,[47] as well as a series of 4-aryltetrahydropyrrolo[3,4-b]indoles.[48] In vivo studies using the rat antiamphetamine model revealed both diastereoisomers and two of the optical isomers of 2 (tentatively assigned the absolute configuration 4aR, 9bR) to be more potent than 1 (30 to 100 x at 24 h), while the most potent pyrroloindole, 3, was comparable to chlorpromazine. Hoechst-Roussel scientists have reported the psychopharmacological profile of the indole 4 (HP-291).[49] This compound, which blocks d-amphetamine but not APO-induced stereotypy in rats, is orally active in blocking conditioned avoidance response (CAR) in animals. It does not appear to have marked α-blocking or anticholinergic properties.



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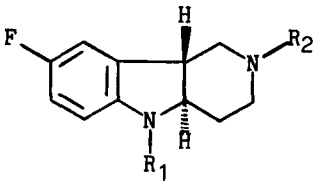

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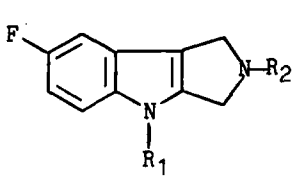
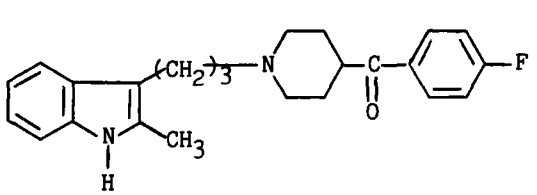


3

1, 2, 3: R_1 = 4-FC_6H_4; R_2 = $(CH_2)_3$CHOH-4FC_6H_4



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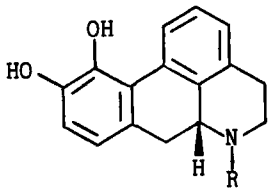


5

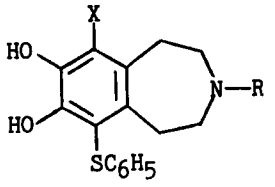
a. R = CH_3 (Apomorphine)

b. R = CH_2CH_2Cl

The concept of selective, irreversible blockade of dopaminergic receptors by means of alkylating ligands is the subject of several related papers.[50-53] In a series of N-(chloroethyl and hydroxyethyl)norapomorphine and norapocodeine derivatives, (-)-N-(chloroethyl)norapomorphine (5b) is clearly the most interesting in biochemical and behavioral studies. It blocks dopamine-sensitive adenylate cyclase in rat striatal homogenates, an effect not overcome by subsequent stimulation of the system by DA.[50,51] This finding suggests a non-competitive, irreversible binding of 5b to the DA receptor. In behavioral studies, 5b (s.c.) antagonizes APO-induced behavioral effects in rodents for up to 5 days.[53] Thus, 5b may represent a novel type of DA receptor antagonist with both pharmacological and clinical utility.

Molecular modification of 17a (SK & F 38,393), a DA agonist on both the peripheral and central nervous systems, has led an SK & F group to a novel class of DA receptor antagonists and neuroleptics.[54] The 6-phenylthio-substituted benzazepines 6a-c compare favorably with chlorpromazine in blocking DA-activated

adenylate cyclase in rat striatum, and each of these compounds blocks the acquisition of an avoidance response in naive rats.[54] The chloro compound 6c is particularly effective in the latter test (20 x chlorpromazine) with no apparent increase in propensity to produce catalepsy. It is noteworthy that both the agonist 17a and the antagonists 6a-c retain a DA substructure within their molecular architectures, albeit not in an extended antiperiplanar disposition.



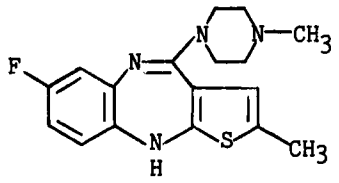
6

a. x = H; R = CH_3

b. x = R = H

c. x = Cl; R = CH_3

Novel tricyclic ring systems containing a pendant aliphatic or heterocyclic amine moiety continue to be rich sources of potential antipsychotic agents. Structure-activity comparisons for a series of 4-piperizinyl-10H-thieno[2,3-b][1,5]-benzodiazepines,[55] as well as for two isomeric 10-piperizinyl-4H-thieno[3,2-b][1,5] and [3,4-b]-[1,5]benzodiazepine systems,[56] have shown that substitution by fluorine or chlorine at position 7 enhances hypothermia in mice and blockade of CAR in rats. The 7-fluoro compound 7 is more potent than clozapine in the latter test yet retains a low propensity to produce catalepsy in rats. Mixed central nervous system activity has been reported for a group of pyrrolobenzodiazepines. For example, 8 shows antidepressant activity as measured by antagonism of tetrabenazine-induced depression in mice, and neuroleptic activity as measured by antagonism of d-amphetamine lethality in aggregated mice.[57] As a prelude to investigating the possible neuropharmacological importance and biological participation of radical cations derived from antipsychotic phenothiazines, Grol et al. have studied in vivo the effect of three series of isomeric thienobenzothiazines on DA metabolism.[58] While quantitative differences exist, all three series of compounds cause a rise in homovanillic acid levels in rat corpus striatum. An open study of 9 (RMI-81,582) in hospitalized male schizophrenics failed to show a correlation of observed clinical improvement with serum prolactin levels.[59] In this regard, 9 behaves like the atypical neuroleptic clozapine. At doses up to 100 mg/day, no cardiovascular or extrapyramidal side effects were noted. Moreover, 9 did not affect white cell blood count. Quantitative pharmacoelectroencephalography has been used to study 10 (SK & F 69,634, clopipazan) in healthy adults.[60] In an uncontrolled clinical study, 10 was found to have a very slow onset of action



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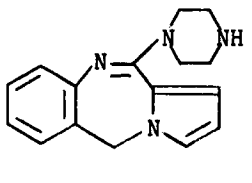

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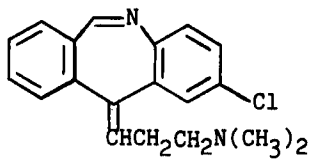

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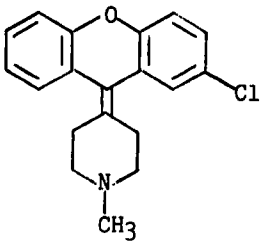

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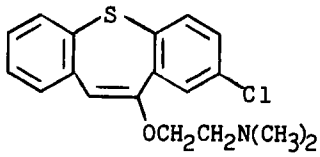

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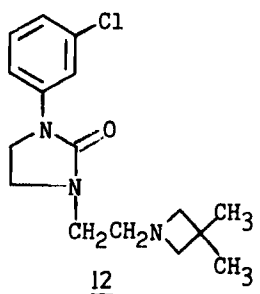


12

and, at doses up to 80 mg/day, did not show antipsychotic potency comparable to established agents. Finally, a series of papers has detailed the behavioral and biochemical pharmacology,[61] pharmacokinetics and metabolism,[62] and toxicity and teratology[63] of 11 (zotepine). Although comparable to chlorpromazine in its psychopharmacological profile, 11 was less active in potentiation of barbiturate sleep and in cardiovascular effects. Compound 11 is structurally related to amethoclothepin, a powerful central nervous system depressant.

A chemically unusual azetidine ring is present in the new, potential neuroleptic agent 12 (DL-308).[64] In a controlled study using healthy volunteers, 12 appeared to be a more potent sedative than thioridazine with less effect on autonomic functions and little effect on motor systems.

This past year has seen a continued interest in the role of centrally active endorphins, enkephalins, and related peptides in the pathogenesis and/or treatment of schizophrenia.[4] Controversial hypotheses have been advanced that implicate both an increase and a decrease of endorphin activity in schizophrenia.[65] Radioimmunoassay of β-endorphin levels of post-mortem human brains has shown no difference, however, between brain peptide content of control and schizophrenic patients.[66] Two recent clinical studies, using a double-blind, placebo-controlled, crossover design, failed to confirm the antipsychotic efficacy of β-endorphin when administered intravenously to schizophrenic patients.[65,67] One of these studies has shown that the method of i.v. administration markedly affects the resulting β-endorphin plasma concentration.[65] The behavioral effects of β-endorphin, as well as related peptides, are the subject of a recent review.[68]

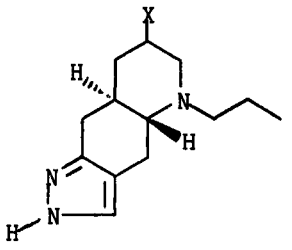
Although previous in vitro studies indicated that the putative antipsychotic β-endorphin fragment des-tyrosine^1-γ-endorphin (DTγE, β-LPH$_{62-77}$) does not act at the neuroleptic receptor labelled by ^{3}H-spiroperidol, in vivo studies now indicate that this compound inhibits ^{3}H-spiroperidol binding in corpus striatum and mesolimbic areas of rat brain.[69] A controlled study of DTγE (2 mg/day, i.m.) in a mixed population of 13 chronic and acute schizophrenic patients, all of whom were maintained on conventional neuroleptic medication, failed to confirm previous encouraging results concerning the efficacy of this peptide in the treatment of chronic schizophrenia.[70] New data have appeared on the behavioral effects of DTγE and α-endorphin (β-LPH$_{61-76}$) in rats.[71] Several β-LPH fragments related to DTγE also have been studied in behavioral test paradigms, and the shortened sequence β-LPH$_{66-77}$ was found to have activity comparable to that of DTγE.[72]

Although neurotensin does not inhibit ^{3}H-spiroperidol binding in either striatal or mesolimbic dopamine systems, intraaccumbens injection of this tridecapeptide antagonizes d-amphetamine-induced rearing and locomotion in rats.[73] This latter finding has led to the hypothesis that neurotensin may be an endogenous neuroleptic-like peptide.[73,74]

Lately, there have been numerous reports concerning the treatment of schizophrenia by hemodialysis.[75] Although controlled trials are rare, a recent limited but controlled study has failed to confirm alleviation of schizophrenic symptomatology by hemodialysis, and the efficacy of such treatment remains, as yet, quite controversial.[76]

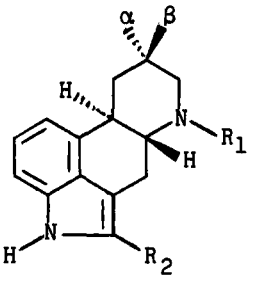
Dopamine Agonists - Based upon a comparison of the absolute configurations of APO with the naturally occurring ergot alkaloids, Lilly scientists have advanced the hypothesis that the rigid pyrroleethylamine moiety of ergoline derivatives, rather than the phenethylamine moiety, accounts for the DA agonist activity of these compounds.[77,78] Inhibition of prolactin release and contralateral rotational behavior in 6-OH-DA nigrostriatal lesioned rats confirmed that several series of bicyclic and tricyclic substructures of the ergoline nucleus, each containing the

putative pyrroleethylamine DA agonist moiety, indeed retained potent dopaminergic activity. Moreover, conversion of the pyrrole ring in these ergoline partial structures to an isosteric pyrazole ring led to compounds such as 13a-b that were comparable in potency to the DA agonist pergolide (14a).[77] Serotonin antagonism and/or α-blocking activity, often seen in ergoline compounds, appears to be absent in the pyrazoles 13a-b.[77] Unexpectedly, a series of tetracyclic pyrazole isosteres of the ergolines (2-azaergolines) were devoid of dopaminergic activity.[79] Rather, one member of this series of 2-azaergolines showed neuroleptic activity in blocking d-amphetamine-L-Dopa induced stereotyped jumping in mice.[79]



13

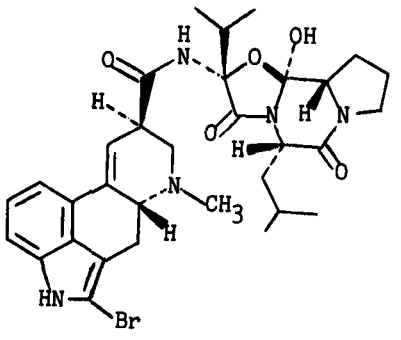
a. x = H
b. x = CH_2SCH_3



14

a. R_1 = n-C_3H_7; R_2 = α = H; β = CH_2SCH_3
b. R_1 = CH_3; α = $NHCON(C_2H_5)_2$; β = R_2 = H
c. R_1 = CH_3; α = H; β = CH_2CN; R_2 = Cl

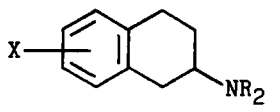
Ergot alkaloids and the synthetic ergolines continue to be examined critically as therapeutically useful DA agonists. Recent biochemical,[80-82] behavioral,[83] and endocrinological[84,85] studies have confirmed the potent DA agonist activity of 14a. Of particular interest is the report that 14a directly stimulates DA-sensitive adenylate cyclase in rat striatal homogenates, an attribute not shared by lisuride (14b), bromocriptine (15), and lergotrile (14c).[80,81,86] The two latter compounds, however, have been shown to have potent in vivo agonist activity at presynaptic DA receptors in both the striatum and the olfactory tubercles.[87] In the case of 15, binding and electrophysiological data suggest that it interacts irreversibly with such central DA receptors.[88] Both 14a and 14b continue to demonstrate encouraging anti-parkinsonian activity in limited clinical trials.[89,90] Unlike APO, neither 15 nor the synthetic ergolene CF 25-397 was efficacious in reducing symptomatology in chronic schizophrenics,[91] nor did they benefit patients with tardive dyskinesia.[92] Finally, an extensive clinical and pharmacological review of 15 is the subject of a recent monograph.[93]



15

Attention has continued to focus on the dopaminergic attributes of 2-aminotetralin derivatives. The nature and number of nuclear substituents, as well as their substitution pattern, and the nature of substituents on the nitrogen atom, greatly influence the pharmacological profiles of such compounds by affecting their regional distribution (ability to penetrate the blood-brain barrier) and locus of action. Behavioral and biochemical studies, selected to differentiate between pre- and postsynaptic DA receptor activation, have now identified 16a (TL-99) as a selective presynaptic DA receptor agonist.[94] Also, Horn has recently

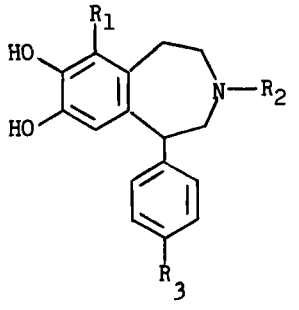
reported a selective stimulation of pre- rather than postsynaptic DA receptors using the 0,0'-dibenzoyl ester of 16b.[95,96] Horn has further extended his studies of dibenzoyl ester pro-drugs to include the 0,0'-dibenzoyl ester of 16c,[96] as well as the N,N-dipropyl analogs.[97] The dopaminergic agonist activities,[98] uptake and storage,[99,100] and metabolism[101] of the pro-drugs of 16b-c are detailed in a series of papers. Interestingly, 16b is a much better substrate than 16c for metabolic degradation by catechol-0-methyltransferase.[101] This finding has been used to explain differences in brain and serum concentrations of the two isomers after i.p. administration in rats. The metabolite 16d is devoid of DA agonist activity.[101] Alterations in striatal DA metabolism, following i.p. administration to rats, have been studied for each of the four positional isomers of monohydroxy-N,N-dipropyl-2-aminotetralin.[102] The 5-hydroxy isomer was the most active in decreasing homovanillic acid levels in the striatum, while the 8-hydroxy isomer was inactive. A Burroughs Wellcome group has shown that 16e is a useful compound for the in vitro study of presynaptic regulation of tyrosine hydroxylase.[103] Finally, a series of 5-hydroxy-2-aminotetralins, in which the ring position ortho to the hydroxyl group is blocked to metabolic hydroxylation by a methyl group, has been examined for DA agonist activity.[104] Compound 16f was comparable to APO in blocking the response to cardioaccelerator nerve stimulation in the cat.



16

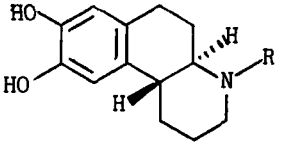
a. x = 6,7-$(OH)_2$; R = CH_3
b. x = 6,7-$(OH)_2$; R = H
c. x = 5,6-$(OH)_2$; R = H
d. x = 6-OH, 7-OCH_3, R = H
e. x = 7-OH, R = n-C_3H_7
f. x = 5-OH, 6-CH_3, R = n-C_3H_7

Separation of central dopaminergic activity and renal vasodilator activity has been reported for the benzazepines 17b-c.[105] The latter compound, SK & F 82526 (17c) is 100x more potent than its parent, SK & F 38393 (17a), in decreasing renal vascular resistance in dogs (i.v.) without appreciably affecting heart rate or arterial blood pressure. Although 17c is an unusually potent stimulant of rat striatal adenylate cyclase (190 x DA), it is virtually inactive when given intraperitoneally in the lesioned rat rotational model. An interesting topographical model of the renal vascular DA receptor has recently been proposed (cf. Chapter 11).[106]



17

a. $R_1 = R_2 = R_3 = H$
b. R_1 = Cl; $R_2 = R_3 = H$
c. R_1 = Cl; R_2 = H; R_3 = OH



18

a. R = CH_3
b. R = C_2H_5
c. R = n-C_3H_7

Cannon et al. have continued their investigations of the DA agonist activity of linearly and angularly annulated octahydrobenzoquinoline derivatives.

The trans ring-fused compounds 18a-c were particularly potent and selective inhibitors of cardioaccelerator nerve stimulation in anesthetized cats (4-25 x APO).[107,108] Moreover, they were uniformly inactive in a battery of tests designed to assess CNS activity. The cis ring-fused isomers of 18, as well as a series of linearly annulated cis and trans ring-fused octahydrobenzo[g]quinolines,[107] and the related series of octahydrobenz[h]isoquinoline derivatives,[109] were virtually devoid of DA agonist activity in both the CNS and the periphery.

N-n-Propyl-N-phenethyl-m-tyramine (RU 24213) and N-n-propyl-di-β-(3-hydroxyphenyl)ethylamine (RU 24926) are two new compounds that have potent dopaminergic activity at the striatal and anterior pituitary levels.[110,111]

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Chapter 3. Benzodiazepine Receptors

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The discovery of high affinity, saturable and stereospecific binding sites for benzodiazepines in the central nervous system (CNS)[1,2] has radically altered our concepts of the molecular mechanisms of benzodiazepine action. The high correlation obtained between the potencies of a series of benzodiazepines in displacing [^{3}H]-benzodiazepines from these binding sites *in vitro* and the potencies of these compounds as anxiolytics, muscle relaxants and anticonvulsants strongly suggest that these binding sites are pharmacologic receptors mediating the therapeutic actions of the benzodiazepines.[1,2] The binding of a drug such as a benzodiazepine or endogenous substance to this recognition site is currently envisioned as initiating a series of events resulting in a pharmacologic or physiologic action. This review describes recent developments on the regulation of the benzodiazepine receptor which have yielded new insights into the modes of action of agents which are structurally dissimilar to the benzodiazepines but share common therapeutic actions. There are several comprehensive reviews on both the chemistry and pharmacology[3,4] of anxiolytic agents (see Chapter 4, this volume) and the benzodiazepine receptor.[5-8] The interested reader is referred to these articles for further detail.

The Benzodiazepine Receptor as Part of a Supramolecular Complex

I. Relationship to GABA Receptors and a Chloride Ionophore. One of the most important advances in probing the molecular pharmacology of the benzodiazepine receptor is the observation that a functional coupling exists between this site and a recognition site for γ-aminobutyric acid (GABA). Although a large body of electrophysiologic, pharmacologic and behavioral (see refs. 5-8 for review) evidence suggests that benzodiazepines facilitate the actions of the inhibitory neurotransmitter GABA, the neurochemical bases for this facilitation remain unclear. Most investigators[9] have not been able to demonstrate an effect of benzodiazepines on binding of [^{3}H]-GABA to GABA receptors, although a recent report[10] suggests that under appropriate conditions *in vitro*, potentiation of [^{3}H]-GABA binding does occur. Nonetheless, the observation that discrete recognition sites for benzodiazepines are present in the CNS has allowed investigators to explore the functional relationship between these two sites. Initial observations on the effects of GABA on [^{3}H]-diazepam binding were negative,[1,11] probably due to the high concentrations of endogenous GABA present in tissue preparations used by these investigators. In extensively washed preparations, a concentration-dependent increase in [^{3}H]-benzodiazepine binding was elicited by GABA and GABAmimetic compounds such as muscimol.[5,12-14] This effect was stereospecifically blocked by the GABA antagonist, bicuculline, and could also be produced *in vivo*[15] by administration of aminooxyacetic acid, a GABA transaminase inhibitor. This increase in [^{3}H]-benzodiazepine binding was the result of an apparent increase in receptor affinity (K_d) rather than an increase in receptor number (B_{max}). These data suggest that the binding of GABA or a GABAmimetic compound to

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a specific recognition site alters the conformation of a benzodiazepine recognition site, resulting in an increase of the apparent affinity of [^{3}H]-benzodiazepines (and presumably endogenous substances) for this site. However, the screening of a series of GABAergic compounds resulted in the observation that there are significant differences in the abilities of GABAmimetic compounds to enhance the binding of [^{3}H]-diazepam and inhibit the binding of [^{3}H]-GABA or [^{3}H]-muscimol. For example, the rigid GABA anlogs THIP and isoguvacine (Fig. 1) are partial agonists, stimulating [^{3}H]-GABA binding. These observations suggest that only a specific population of GABA receptors may be involved in the regulation of benzodiazepine receptor affinity.[16,17] Furthermore, regional differences in the CNS with respect to the enhancement of [^{3}H]-diazepam binding by GABA also suggest that not all benzodiazepine receptors are functionally coupled to GABA receptors.[18] These findings also imply that drugs and/or endogenous substances may affect the regulation of the benzodiazepine receptor not only by direct (usually competitive) occupation of a "recognition site" for benzodiazepines, but also by means of a GABA-benzodiazepine receptor coupling mechanism.

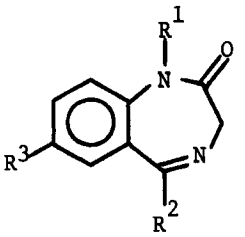
The observation that anions, such as chloride and iodide (but not maleate, citrate, or sulfate), also affect (increase) the affinity of [^{3}H]-benzodiazepines[19] (and presumably endogenous ligands) for the benzodiazepine receptor suggests that the regulation of this receptor is far more complex than initially believed. It is now hypothesized that there is a functional coupling of a benzodiazepine recognition site to both a GABA recognition site and an associated chloride ionophore. Electrophysiological observations following the application of benzodiazepines to the CNS [either *in situ* (*e.g.* by iontophoresis) or *in vivo* (*e.g.* by parenteral administration)] have consistently supported an interaction of benzodiazepines with both a GABA receptor and a chloride ionophore.

The functional association of benzodiazepine receptors with a chloride ionophore has been further strengthened by neurochemical findings from several laboratories. O'Brien and Spirt[20] have recently demonstrated that the convulsant benzodiazepine Ro-5-3663 (Fig. 1) is more potent as an inhibitor of GABA-enhanced benzodiazepine binding than in inhibiting "basal" (non GABA-stimulated) binding. Several drugs, as well as endogenous factors,[21,22] have now been found to possess similar characteristics (*e.g.* caffeine, inosine, meprobamate and a low molecular weight, non-purine endogenous factor). These data support the hypothesis that the benzodiazepine receptor may be affected by compounds interacting at loci other than the benzodiazepine recognition site. Many of the compounds demonstrated to antagonize the GABA-stimulated binding of [^{3}H]-benzodiazepines may not do so at a GABA recognition site, since compounds such as Ro-5-3663 do not inhibit [^{3}H]-muscimol or [^{3}H]-GABA binding.[20,23] However, Olsen and his colleagues[23,24] have demonstrated that many compounds which bind to a benzodiazepine recognition site with a relatively low affinity are far more potent as inhibitors of [^{3}H]-α-dihydropicrotoxinin (DHP) binding. This compound is a derivative of the convulsant picrotoxin, which has been proposed to exert its effects by interaction with sites controlling the flux of chloride ions, referred to as the chloride ionophore.

II. Relationship to the Action of Barbiturates and GABA. Among the compounds which Olsen *et al.*, have demonstrated to displace DHP binding are many pharmacologically active barbiturates (Fig. 1). These compounds might then affect the benzodiazepine receptor by an allosteric mechanism. In the initial studies on characterization of the benzodia-

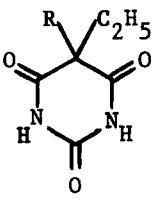
zepine receptor, pentobarbital was reported not to affect [^{3}H]-diazepam binding.[1,11] However, one of these studies[1] used concentrations of pentobarbital (3 μM) below those observed in tissue after pharmacologically relevant doses,[25,26] while the other study reported no inhibitory effects. We[27-29] have recently examined the effects of pentobarbital on [^{3}H]-diazepam binding and have observed two distinct actions on the benzodiazepine receptor. At relatively high concentrations, pentobarbital increases the "basal" (EC_{50} ~220 μM) binding of [^{3}H]-diazepam to brain membranes which have been washed free of endogenous GABA. Pentobarbital also potentiates GABA-enhanced binding of [^{3}H]-diazepam at approximately 10-fold lower concentrations. The former phenomenon appears to be anion dependent, since both the maximum increase elicited by pentobarbital as well as the EC_{50} in the absence of chloride ion are depressed. It should be noted that maximum stimulatory concentrations of GABA are not potentiated by pentobarbital; at these concentrations of GABA, the effects of the two agents become nearly additive. The phenomenon of increased [^{3}H]-diazepam binding appears to be due to changes in receptor affinity, rather than receptor number. In the presence of pentobarbital (± GABA), the apparent affinity for [^{3}H]-diazepam (and presumably for endogenous ligands of the receptor) is increased. These effects were antagonized by both picrotoxin[23] and bicuculline.[28,29] The observation that both bicuculline and picrotoxin are capable of antagonizing pentobarbital-enhanced [^{3}H]-diazepam binding may reflect the interdependence of a benzodiazepine recognition site with both a GABA receptor and a chloride ionophore. These data appear to correlate well with the electrophysiological effects of pentobarbital which has been shown to potentiate GABA-mediated activation of chloride ion conductance and, at higher concentrations, to activate chloride conductance alone.[30-32] The concentrations of pentobarbital necessary to potentiate GABA-enhanced binding of [^{3}H]-diazepam correspond to plasma concentrations following anxiolytic/sedative doses of drug, while concentrations needed to enhance "basal" [^{3}H]-diazepam binding correspond to concentrations observed after anesthetic doses of pentobarbital. However, it is premature to assign either electrophysiologic or pharmacologic actions to these neurochemical events, since there are many experimental findings which are not yet reconciled with this hypothesis. For example, we have observed that phenobarbital, which also possesses sedative, anesthetic and anticonvulsant properties, does not appear to significantly enhance [^{3}H]-diazepam binding under conditions where pentobarbital elicits a robust effect. Other pharmacologically active barbiturates, such as secobarbital and hexobarbital, do appear to enhance binding.[33] Barbiturates containing an asymmetric center exhibit stereospecificity, but the difference between the (+) and (-) enantiomers is only 3-4 fold, far less than enantiomers of a benzodiazepine at the benzodiazepine receptor[2] or of the enantiomers of propranolol at the β-receptor. Hydroxypentobarbital, the pharmacologically inactive metabolite of pentobarbital, does not enhance [^{3}H]-diazepam binding. Furthermore, the convulsant barbiturate dimethylbutylbarbituric acid (DMBB) is also effective in enhancing [^{3}H]-diazepam binding. This compound may produce pharmacologic actions other than seizures, and the behavioral actions of low doses of this compound have not been studied in sufficient detail[34] to determine whether DMBB shares common pharmacologic actions with other barbiturates. Obviously, further investigation is needed to determine if such changes in the benzodiazepine receptor affinity represent a pharmacologically relevant phenomenon.

III. Relationship of Other Compounds to the Benzodiazepine Receptor. Pyrazolopyridines, such as SQ 20,0009 (etazolate), SQ 65,396 (cartazolate) and ICI 136,753 (Fig. 1), have also been demonstrated to enhance



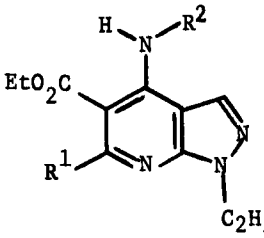
Benzodiazepines:

	Diazepam	Ro 5-3663
R^1=	CH_3	H
R^2=	C_6H_5	CH_3
R^3=	Cl	H



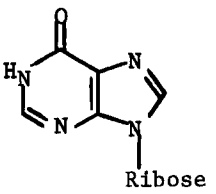
Barbiturates:

Pentobarbital	R= $CH(CH_3)(CH_2)_2CH_3$
Phenobarbital	R= C_6H_5
DMBB	R= $CH(CH_3)CH_2CH(CH_3)_2$

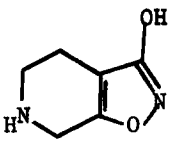


Pyrazolopyridines:

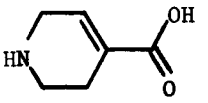
	R^1	R^2
SQ 20009	H	$N=C(CH_3)_2$
SQ 65396	H	n-C_4H_9
ICI 136,753	CH_3	n-C_4H_9



Inosine



THIP



Isoguvacine

Figure 1

[^{3}H]-diazepam binding. They exert this effect, like barbiturates, by increasing the apparent affinity of radiolabelled benzodiazepines for the benzodiazpine receptor.[35,36,37] These compounds have been reported to have anxiolytic[38] actions with no anticonvulsant and minimal sedative effects. There are several similarities between their effects on the benzodiazepine receptor and those of barbiturates, such as pentobarbital. The stimulatory effect of these pharmacologically active pyrazolopyridines on [^{3}H]-benzodiazepine binding appears to be dependent upon chloride ion and is blocked by both picrotoxin and bicuculline.[37,39,40] Furthermore, they displace [^{3}-H]-α-DHP from sites presumed to be at the chloride ionophore, with potencies comparable to those required to enhanced [^{3}H]-benzodiazepine binding.[23] A combination of maximal concentrations SQ 20,009 and pentobarbital did not enhance binding to a significantly greater extent than did a maximal concentration of either agent alone,[23] which suggests a common locus of action. This hypothesis is further supported by the reported dual actions of SQ 20,009, the direct effect previously described, and a potentiation of GABA and

muscimol-enhanced [^{3}H]-benzodiazepine binding. The mechanism by which the increases in [^{3}H]-benzodiazepine binding by pyrazolopyridines occur is still under investigation. However, these compounds increase the number of [^{3}H]-GABA or muscimol binding sites *in vitro*.[41,42] This increase in GABA receptor number at 0° suggests this phenomenon may be due to an "unmasking" of receptors by removal of an endogenous inhibitor(s) of GABA from the membrane. This increase in receptor number does not occur if membranes have been pretreated with the detergent Triton-X,[41] which further supports the hypothesis that an endogenous inhibitor may be removed. One notable difference between the neurochemical effects of barbiturates and pyrazolopyridines is that although pentobarbital has also recently been demonstrated to enhance GABA binding,[43] this effect occurs by increasing the apparent affinity of the GABA receptor (the high, not the low affinity binding component), rather than by altering receptor number. This difference might suggest that several populations of chloride ionophores are being activated by different drugs, since this effect is also picrotoxin-sensitive.[43] The lack of additivity of maximal concentrations of pyrazolopyridines and pentobarbital might argue against this hypothesis; certainly, further investigation is necessary to compare the neurochemical actions of these compounds.

Ethanol has also been recently demonstrated to affect [^{3}H]-benzodiazepine binding.[44] At concentrations between 10-100 mM (corresponding to plasma concentrations of 46-460 mg%, which are well within the range observed following moderate to large doses of ethanol), an increase in [^{3}H]-diazepam binding was observed. A potentiation of the effects of submaximal concentrations of muscimol was also observed, suggesting that like barbiturates and pyrazolopyridines, ethanol has a dual action on the benzodiazepine receptor. These effects are analogous to the electrophysiological effects of ethanol recently described by Nestoros[45] who demonstrated an interaction of ethanol with GABAergically-mediated inhibitory synaptic transmission. No details on the effects of ions, channel blockers or GABA antagonists have been reported.

IV. Benzodiazepine Receptor Heterogeneity. Initial studies characterizing the benzodiazepine receptor suggested a single class of binding sites. Scatchard analyses of binding data with either [^{3}H]-diazepam or flunitrazepam yielded linear plots with no tendency to resolve into multiple components. Hill coefficients obtained by displacement of radiolabelled benzodiazepines with other nonradioactive benzodiazepines were near unity.[46] Furthermore, the K_d of [^{3}H]-diazepam did not vary significantly between different brain regions,[47] further supporting the notion that there is a single population of receptors. However, recent observations from several laboratories have provided strong evidence suggesting there are heterogeneous populations of benzodiazepine receptors in the CNS. Displacement of [^{3}H]-benzodiazepines from receptor sites by two groups of compounds, the triazolopyridazines[48] (a new class of anxiolytic/anticonvulsant) and β-carbolines,[49] yielded Hill coefficients significantly lower than unity, suggesting a negative cooperativity between multiple receptor subtypes. Regional differences in the potencies of these compounds have also been reported.[49-50] The use of novel benzodiazepines[51] containing functional alkylating groups has also supported the existence of benzodiazepine receptor subtypes. The use of photoaffinity labelling has provided additional evidence for a heterogeneity of receptors. Using this technique, Seighart et al.[52] have demonstrated the presence of multiple species of benzodiazepine receptors which have been photoaffinity labelled with [^{3}H]-flunitrazepam. There appear to be marked regional differences in the number of species

obtained following gel electrophoresis of these receptors; however, the apparent molecular weight of the different bands (determined by mobility on polyacrylamide slab gel electrophoresis) varied between 51,000 and 59,000 daltons. The presence of heterogeneous populations of benzodiazepine receptors suggests that a particular type may be involved in a specialized role (e.g. mediation of anticonvulsant action). This hypothesis is supported by observations with the triazolopyridazines, which appear to have differential affinities for receptor subtypes, and possess anxiolytic and anticonvulsant actions with a minimal sedative liability.

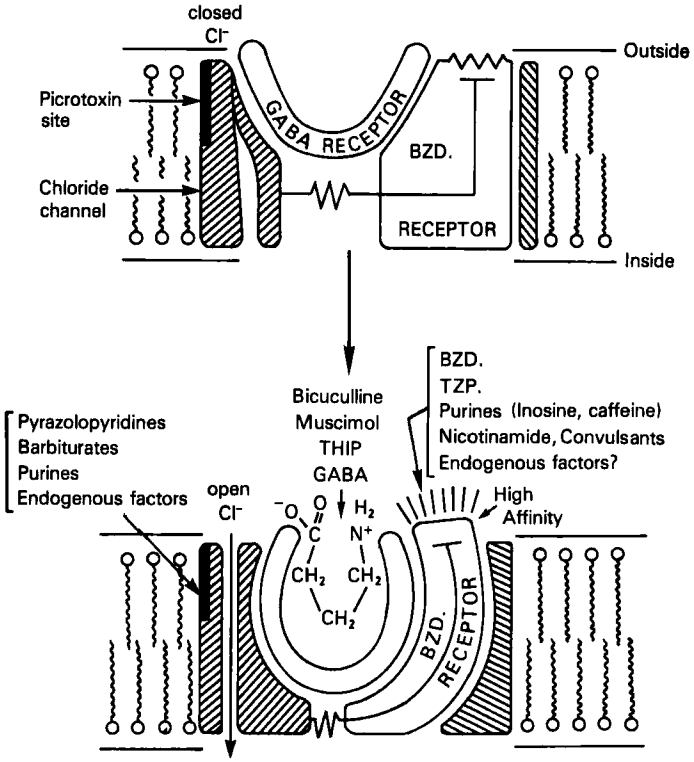


Figure 2.
Stochastic Model of a
Benzodiazepine-GABA Receptor-Chloride Ionophore Complex

Abbreviations: BZD, benzodiazepines; TZP, triazolopyridazines; THIP, 6,7-tetrahydroisoxazolo-5,4,-c-pyridin-3-ol; GABA, γ-aminobutyric acid.

A Stochastic Model for Regulation of the Benzodiazepine Receptor

The data obtained within the past three years have afforded sufficient information for constructing a stochastic model governing "dynamic" regulation of the benzodiazepine receptor (Fig. 2). It is now apparent that the benzodiazepine receptor [a recognition site for benzodiazepines, endogenous compounds (e.g. purines), triazolopyridazines,[48] β-carbolines[49] and other compounds] is coupled to a GABA receptor (a recognition site for GABA, muscimol, bicuculline and other compounds) and a chloride ionophore (a recognition site for barbiturates, pyrazolo-

pyridines, picrotoxin derivatives and endogenous compounds). The question of whether these components are physically as well as functionally coupled is still unresolved.[53,54] However, the observation that under certain solubilization conditions, the GABA-enhancement of [^{3}H]-benzodiazepine binding to the solubilized "receptor" is lost, suggests that these recognition sites are not components of the same protein. If they were, it is very likely the GABA-enhancement would be retained. Nonetheless, it is hypothesized that the binding of a drug or endogenous compound to either the GABA receptor or choride ionophore can allosterically modify the benzodiazepine receptor resulting in an altered affinity of [^{3}H]-benzodiazepines (and presumably endogenous ligands) for this site. The abilities of both pyrazolopyridines and barbiturates to increase the apparent affinity of [^{3}H]-benzodiazepines appear to be ion (viz. chloride) dependent while GABA (and muscimol) enhancement is not affected by the absence of chloride ion. The observation that both the barbiturate and pyrazolopyridine enhancements of benzodiazepine receptor affinity are blocked by both picrotoxin and bicuculline would suggest that the GABA receptor must be in an "open" conformation (no antagonist present) for the binding of either a barbiturate or pyrazolopyridine to the chloride ionophore (i.e. α-DHP binding site) to result in an affinity change in the benzodiazepine receptor. Hence, we propose a functional linkage between these sites.

With this model, it is possible to predict that a drug may elicit its pharmacologic actions by direct occupation[55] of the benzodiazepine recognition site or by allosteric interaction with another component of this benzodiazepine/GABA/chloride ionophore receptor complex. This model offers a working hypothesis to explain the neurochemical phenomena outlined in this review, and could ultimately explain electrophysiological and behavioral effects of several psychotropic agents. This model has proven useful in analysis of the behavioral, electrophysiological and neurochemical actions of inosine (Fig. 1), which we have proposed[5,6] to be an endogenous ligand of the benzodiazepine receptor. Electrophysiological studies with inosine using primary cultures of fetal mouse spinal cord revealed two types of transmitter-like effects: a rapid, desensitizing excitatory response and a non-desensitizing inhibitory response. The benzodiazepine flurazepam (Dalmane®) produced a similar excitatory response, which cross-desensitized with the inosine response. Flurazepam also blocked the inhibitory response. These results suggest that inosine activates at least two different conductances on spinal neurons; it appears to have a benzodiazepine-like effect at one of these loci and to antagonize a benzodiazepine action at the other. Pharmacologic studies with relatively large doses of inosine administered either intracerebroventricularly[55,56] or peripherally[57] revealed a benzodiazepine-like effect. That is, inosine appeared to partially antagonize or reverse pentylenetetrazole (Metrazole®) or caffeine-induced seizures. However, doses of inosine which were approximately 10-fold lower than those needed to antagonize the convulsant actons of caffeine disrupted the behavioral[58,59] and anticonvulsant actions of diazepam. Inosine has a relatively low affinity (K_i of 800-900 μM) for the benzodiazepine recognition site but is approximately 10-fold more potent in antagonizing both GABA-enhanced [^{3}H]-diazepam binding[21] and in inhibiting [^{3}H]-α-dihydropicrotoxin binding.[24] It is proposed that relatively low concentrations of inosine bind to the chloride ionophore site resulting in an apparent reduction in benzodiazepine receptor affinity. This might result in a disruption of the effects of benzodiazepines both electrophysiologically and behaviorally. Higher concentrations of inosine would, in addition, bind directly to the benzodiazepine receptor (overcoming the former action of inosine), which would elicit benzodiaze-

pine-like effects, both behaviorally and electrophysiologically. At present, the physiological role of inosine in regulating this supramolecular complex is unknown.

The pharmacologic "thread," which is common to the actions of barbiturates, pyrazolopyridines and ethanol, is an anxiolytic action. Ethanol is man's oldest and most widely used anxiolytic. Prior to the introduction of benzodiazepines, barbiturates were widely used as anxiolytics and continue to be prescribed occasionally for this purpose. The pyrazolopyridines, demonstrated to have anxiolytic actions in experimental animals, must be given a comprehensive clinical trial to determine their efficacy as anxiolytics in man.

Nonetheless, it is still premature to attempt to assign a pharmacologic action to specific changes in a benzodiazepine/GABA receptor/chloride ionophore complex. For example, phenobarbital, which has anxiolytic actions, does not appear to enhance [^{3}H]-diazepam binding with a potency or efficacy (i.e. EC_{50} or maximal response) consonant with either electrophysiological or behavioral studies. Furthermore, the finding that the convulsant barbiturate DMBB, which has not been shown to have an anxiolytic action, is a potent activator of [^{3}H]-diazepam binding is another apparent inconsistency which remains to be resolved in the context of this stochastic model. These observations underscore our lack of complete information on the electrophysiological and behavioral effects of many of these compounds and suggest the need for further studies to clarify the role and regulation of the benzodiazepine receptor as part of a supramolecular complex.

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Chapter 4. Anti-Anxiety Agents, Anticonvulsants, and Sedative-Hypnotics

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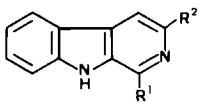
Introduction - Although the benzodiazepines (BZs) have been in widespread use for some 20 years, it is only in the past few years that rapid advances have been made in understanding the neurochemical mechanisms responsible for their therapeutic actions. This recent research has generated not only exciting results in the biochemical, electrophysiological, pharmacological and behavioral areas, but also exciting new opportunities for the medicinal chemist.

Mechanism of Action - It is now apparent that highly specific recognition sites exist in the brain for the BZs.[1-7] Detailed biochemical studies of the CNS BZ receptors have suggested that more than one subclass of such receptors may exist.[8,9] Autoradiographic methods have been used to localize BZ receptors in rat brain.[10-12]

The "GABA hypothesis" relating to the actions of BZs in the brain continues to gain support. The psychopharmacological effects of the BZs may be due, at least in part, to their ability to potentiate and prolong the synaptic actions of the inhibitory amino acid GABA.[13-18] The existence of a novel bicuculline-insensitive GABA receptor, which is different from the conventional postsynaptic inhibitory GABA receptor, has been reported.[19] It is suggested that the GABA recognition sites associated with BZ receptors may represent a separate subclass of CNS GABA receptors.[8,20] Certain BZ receptors appear to be associated with GABA receptors and/or ion recognition sites yielding multiple receptor complexes,[13,21-25] of which the different components interact allosterically,[26] possibly inducing conformational changes in the BZ receptor.[27,28] The high and low affinity GABA binding sites may correspond to BZ receptor-linked and non-BZ receptor-linked GABA receptors, respectively.[29]

The search for an endogenous substance in the brain interacting specifically with the BZ receptor has led to the identification of several possible candidates. The isolation of high molecular weight protein modulators has been reported.[13,22,30-31] Certain purines, such as hypoxanthine and inosine,[32-34] and nicotinamide[35] have been found to interact weakly with CNS benzodiazepine binding sites and have, to a certain extent, diazepam like activity in vivo.

The γ-substance isolated from human urine and animal brain has been identified as ethyl β-carboline-3-carboxylate (β-CCE, 1a). It displaces ^{3}H-diazepam (2a) from specific receptors with an IC_{50}=4-7nM, compared to approximately 5nM for lorazepam.[36] The presumed endogenous ligand for BZ receptors may be a derivative of β-CCE,[37,38] because β-CCE is not present as such in any measurable concentration in brain or peripheral tissues in vivo. Interestingly, β-CCE has been shown to potentiate pentylenetetrazol (PTZ) and maximal electroshock (MES)-induced convulsions in vivo, and to antagonize the effects of diazepam, both in vivo and in vitro.[39,40]



1a, R^1 = H, R^2 = $CO_2C_2H_5$
1b, R^1 = H, R^2 = CO_2CH_3
1c, R^1 = H, R^2 = CO_2H
1d, R^1 = CH_3, R^2 = $CO_2C_2H_5$
1e, R^1 = CH_3, R^2 = H
1f, R^1 = H, R^2 = H

Evidence has been presented that supports the hypothesis that the anti-conflict action of BZs may also be mediated by serotonin, possibly with the involvement of GABA,[38,41] and that BZs exert their antianxiety action by interfering with serotonergic systems in the brain.[4,42] Dopamine has also been implicated in the action of BZs.[43,44]

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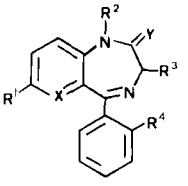
It has been suggested that adenosine may play an important role in the action of BZs. BZs inhibit adenosine uptake into rat brain synaptosomes.[45-48] In addition, adenosine uptake blockers [e.g., dipyridamole (19)] inhibit ^{3}H-diazepam binding to rat brain cortical synaptosomal membranes.[49]

Animal Models - Because tests such as the Geller-Seifter procedure require long and regular testing of experimental animals,[50] there remains a need for the development of specific and simple animal behavior tests. In conflict procedures in which food or water is used as a reward, agents affecting the consummatory drive mechanisms can show up as false positives.[51] Simple animal behavior models for the anxiolytic effects of benzodiazepines have been reported (increased exploratory behavior, social interactions).[52-54] A new approach utilizes the locus coeruleus(LC)-norepinephrine connection with anxiety. Stimulation of the LC by electrodes causes anxious behavior which can be blocked by diazepam.[55-56] The selective antagonism of a PTZ discriminative stimulus by anxiolytics may also serve as a useful animal model for the detection of anxiolytic activity.[57] The effect of diazepam on extinction-induced aggression showed that BZs do not act on aggressiveness per se, but rather strengthen the prevailing behavioral attitudes in the animal at the time of testing.[58]

Benzodiazepines and Related Compounds

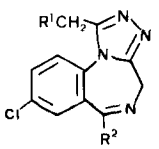
Anxiolytic Agents - Systematic reviews of BZs in clinical use have recently appeared,[59-64] as have clinical studies on the treatment of anxiety states with alprazolam (3a),[65-67] camazepam (2b),[68] clobazam (4),[69,70] clotiazepam (Y 6047, 5),[71] cloxazolam (MT 14-411, 6),[72] lopirazepam (D12524, 2c),[73] ketazolam (7)[74-76] and prazepam (2d).[77] Alprazolam (3a) was equally effective as imipramine in treating depression.[78] In comparative behavioral studies in monkeys, clobazam (4) (0.5-25 mg/kg) produced no impairment of a performance task (spatial delayed alternation), while the triazolo-1,4-thienodiazepines brotizolam (8a) and WE 973 (8b) had pronounced effects (0.5-3 mg/kg). Temazepam (2e) showed effects only in



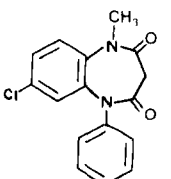
2

	R^1	R^2	R^3	R^4	X	Y
2a	Cl	CH_3	H	H	CH	O
2b	Cl	CH_3	$OCON(CH_3)_2$	H	CH	O
2c	Cl	H	OH	Cl	N	O
2d	Cl	CH_2	H	H	CH	O
2e	Cl	CH_3	OH	H	CH	O
2f	Cl	$(CH_2)_2N(Et)_2$	H	F	CH	O
2g	NO_2	CH_3	H	F	CH	O
2h	Cl	CH_2CF_3	H	F	CH	S
2i	NO_2	H	H	H	CH	O
2j	NO_2	H	H	Cl	CH	O
2k	Cl	H	OH	Cl	CH	O

the higher dose range (5-25 mg/kg).[79] A QSAR study on a series of BZs attempted to correlate chromatographic Rm values with behavioral activity.[80]



3a, R^1 = H, R^2 = phenyl
3b, R^1 = N(CH$_3$)$_2$, R^2 = phenyl
3c, R^1 = H, R^2 = piperidino
3d, R^1 = H, R^2 = 2-chlorophenyl



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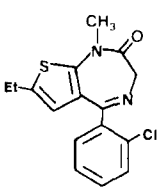

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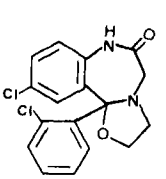

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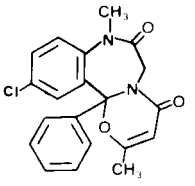

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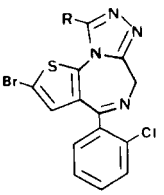
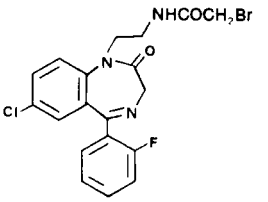


8a, R = CH_3
8b, R = C_6H_{11}



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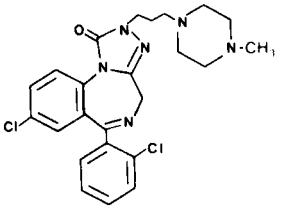
Kenazepine (9) was used to alkylate BZ receptors.[81] Chemical modifications of alprazolam (3a) lead to triazolo-1,4-BZs with interesting psychotropic activity; in particular, 3b[82] and 10[83] have a pharmacological



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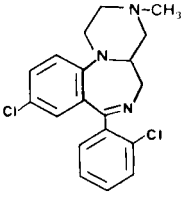

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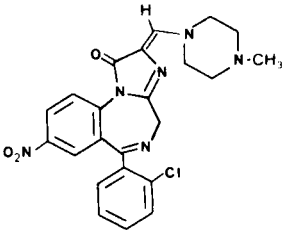


12

profile which suggests that they may have a useful combination of antidepressant and antianxiety activity. The anxiolytic activity of 3c has been described.[84] Substitution at C-4 of 3a diminished anxiolytic activity.[85] Compound 11 showed modest antagonism against PTZ induced seizures and weak activity in the diazepam binding assay.[86]

Sedative-Hypnotics - The hypnotic activity of HR 158 (12) has been confirmed in humans at doses of 0.5, 1.0 and 2.0 mg, with no significant impairment of early morning psychomotor performance following nocturnal medication.[87]

Triazolam (3d) has been compared with flurazepam (2f) in hospitalized psychiatric patients,[88] and with flunitrazepam (2g) in healthy subjects[89] and preoperative patients,[90] and found to be an effective sleep inducer with few side effects. After one year of continuous therapy, triazolam continued to display excellent hypnotic properties.[91] Its metabolism and disposition have been studied.[92] Brotizolam (8a) was studied in normal subjects at 0.2 and 0.4 mg and found to be an effective short acting hypnotic essentially free of adverse effects on sleep and residual effects on performance.[93] Quazepam (Sch 16134, 2h) was studied at 15 and 20 mg in chronic insomniacs and was found to be an effective sleep inducer with no side effects or rebound insomnia on withdrawal.[94,95] Temazepam (2e) at 40 and 60 mg was a potent hypnotic in normal subjects, but produced a disruption of psychomotor performance and pronounced morning hangover.[96] At 20 mg, however, hypnotic activity was retained with no adverse effects noted.[97] The hypnotic effectiveness of ketazolam (7) has been studied in anxious subjects.[98] Both clonidine and nitrazepam (2i) exhibited similar sedative and hypotensive effects in clinical evaluation, apparently resulting from a central reduction in sympathetic tone.[99]

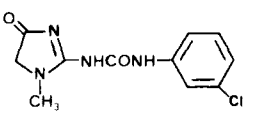
Anticonvulsants - Intravenously administered clonazepam (2j) was shown to be effective and devoid of serious side effects in the treatment of status

epilepticus in children.[100] The use of lorazepam (2k) in the treatment of status epilepticus has also been discussed.[101]

A study of the interaction of phenytoin (33a) and diazepam (2a) in the CNS has indicated that one site of action of phenytoin in the brain is the BZ binding site, and that this binding site may be relevant to convulsant-anticonvulsant activity. Phenytoin was found to enhance specific binding of BZ by increasing the total number of BZ binding sites.[102]

Non-Benzodiazepines

Anxiolytic Agents - While a six-month uncontrolled study indicated that fenobam (13) was effective in relieving symptoms of anxiety in patients,[103] a double-blind one-month controlled study did not confirm this.[104] The peptidoaminobenzophenones are a novel class of ring-opened derivatives of 1,4-BZs.[105,106] Compound 14 antagonized PTZ induced convulsions (ED_{50}=0.56 mg/kg po, mice) and foot-shock induced fighting, and produced sedation. It is weakly active in the ^{3}H-diazepam binding assay,



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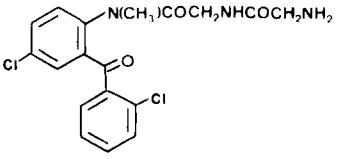

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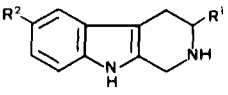
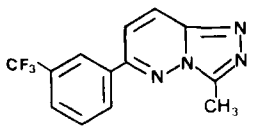


15a, $R^1 = CO_2CH_3$, $R^2 = H$
15b, $R^1 = CO_2C_2H_5$, $R^2 = H$
15c, $R^1 = H$, $R^2 = H$
15d, $R^1 = H$, $R^2 = OCH_3$

but is converted into compounds having a high affinity for BZ receptors by incubation with crude synaptosomes from rat brain or rat liver homogenates.[107,108] Identification of metabolites suggests that these peptidoaminobenzophenones function as prodrugs and are hydrolyzed enzymatically and then cyclized to give biologically active 1,4-BZs.[107-110]

Studies on β-CCE (1a) and derivatives have shown the methyl ester 1b to be as active as β-CCE in ^{3}H-flunitrazepam (^{3}H-FNM) binding, whereas the free acid 1c, the tetrahydro derivatives 15a,b, and the 1-methyl analog 1d were nearly inactive. Harmane (1e) and norharmane (1f) are the most potent β-carboline inhibitors of ^{3}H-FNM binding found to date in the brain and urine of humans and rats (IC_{50}=7.2 and 8.2 μM, respectively).[111]

The hypothesis that purine analogs may function as endogenous ligands or modulators of the BZ receptor is supported by the activity of derivatives such as the triazolopyridazine CL 218, 872 (16), now a lead compound in the search for structurally new anxiolytics. CL 218,872 binds to the ^{3}H-diazepam receptor (K_I=100nM), is active in the water-lick conflict procedure, has only weak sedative effects and does not potentiate alcohol-induced narcosis.[3,4,8,32] While BZs have similar affinities for Type 1 (BZ_1) (anxiolytic) and Type 2 (BZ_2) (sedation, ataxia) receptors, 16 has high affinity for only Type 1.[112,113] A novel purine EMD 28422 (17) enhances the binding of ^{3}H-diazepam to the BZ receptor in vivo by increasing the number of BZ receptors rather than the affinity. It has a psychopharmacological profile similar to the BZs.[114] 1-Methylisoguanosine (18), a muscle relaxant, and dipyridamole (19), an adenosine uptake blocker, are active in the ^{3}H-diazepam binding assay (IC_{50}=19μM and 300nM, respectively).[115] Inosine is reported to reverse the increase in exploratory activity induced in mice by diazepam.[116]



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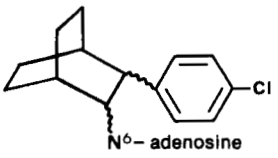
In vitro experiments on a BZ receptor membrane preparation showed a



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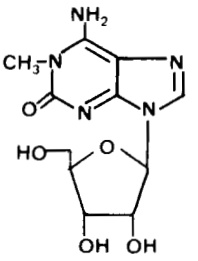

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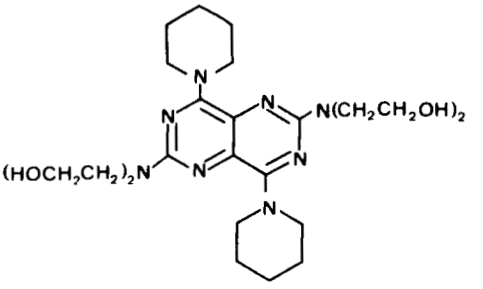

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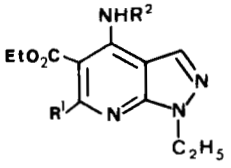
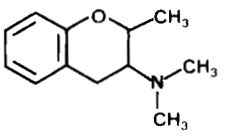


20a, $R^1 = H$, $R^2 = n\text{-}C_4H_9$
20b, $R^1 = H$, $R^2 = N = C(CH_3)_2$
20c, $R^1 = CH_3$, $R^2 = n\text{-}C_4H_9$

modulation of GABA receptor binding by the anxiolytic pyrazolopyridines cartazolate (SQ 65396, 20a) and etazolate (SQ 20,009, 20b).[117,118] They do not bind directly to the BZ receptor, but enhance the binding of diazepam. While these compounds exhibited only weak activity in the rat conflict test, they were reportedly active in pilot clinical trials.[4] Tracazolate (ICI 136,753, 20c)[119] exhibits anticonflict activity in shock-induced suppression of drinking in rats (MED=20 mg/kg po), and weak to moderate activity as an antagonist of PTZ seizures in mice, but differs from the BZs in that it produces no sedation or muscle relaxation.[120] A double-blind study indicated that trebenzomine (21) is effective as an antidepressant-anxiolytic.[121] The 5-HT uptake inhibitor zimelidine (22) blocks aggressive behavior in isolated male mice.[122] In a double-blind study buspirone (23) produced significant antianxiety effects



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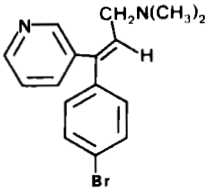

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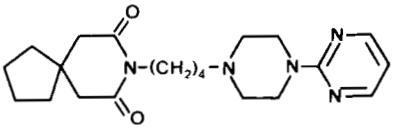
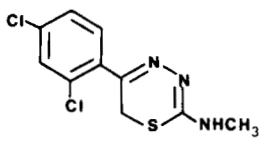


23

similar to those of diazepam but with fewer side effects. It showed no anticonvulsant action and produced no sedation. Buspirone neither inhibits nor facilitates BZ binding to rat cortical membranes.[123] Although the thiadiazine RMI 117,617A (24) has a pharmacological profile like the BZs, it has no effect on ^{3}H-flurazepam binding to rat brain membranes.[124] The tetrahydro-2(1H)-pyrimidone 25 has both antidepressant and anxiolytic activity.[125] Isolation, synthesis and pharmacology of metabolites of 25 have been reported.[126] From a series of hexahydropyridooxazinequinoxalines, 26 was reported to inhibit diazepam binding in rat brain membranes,



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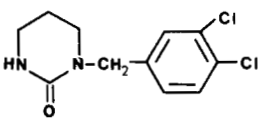

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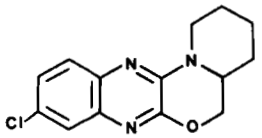

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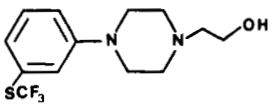
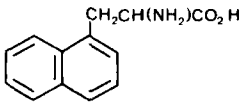


27

with an IC_{50}=14nM.[127] In the 4-plates test in mice, 27 showed +118% disinhibition at 0.3 mg/kg po (chlordiazepoxide: +86%/3 mg).[128]

Sedative-Hypnotics - A naphthyl analog 28 of tryptophan has been reported to be a sleep inducer in animals.[129] Sedative activity was found in a



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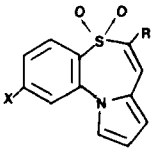
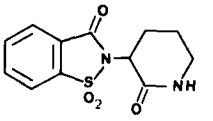


29a, X = Cl, R = 3-pyridyl
29b, X = Cl, R = 4-$NO_2C_6H_4$
29c, X = OCH_3, R = C_6H_5



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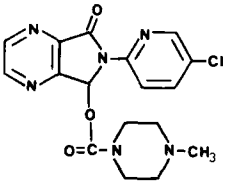
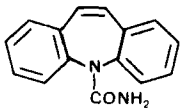


31

series of pyrrolo[2,1-d][1,4]benzothiazepin-6,6-dioxides (29a,b,c), with 29a reported to have activity superior to that of diazepam when tested in rats against amphetamine-induced motor activity.[130] In dosages up to 200 mg, supidimide (CG-3033, 30) was found to be an effective hypnotic without morning hangover effects or ataxia. Computer-analyzed EEG indicated that its mode of action is different from that of flurazepam.[131] The hypnotic effect and tolerance of zopiclone (31) were evaluated in preoperative hospitalized patients in a comparative study with nitrazepam, flurazepam and flunitrazepam. Flunitrazepam was found to be superior to the other drugs tested, which were nearly equally effective.[132] A review of thiophene derivatives in pharmaceutical research has included hypnotics, sedatives, muscle relaxants and anxiolytics.[133]

Anticonvulsants - A comprehensive review of epilepsy and antiepileptic drugs has recently been published,[134] as well as somewhat more abridged reviews of currently used anticonvulsants.[135,136]

When used in combination with carbamazepine (CBZ, 32) or phenytoin (33a), valproic acid (34) serum levels were found to be low, presumably



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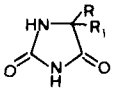
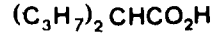


33a, R, R_1 = C_6H_5
33b, R = CH_3, R_1 = C_2H_5



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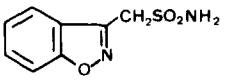
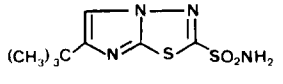


35

due to enhanced metabolism.[137] Additional studies on binding and binding interactions of these anticonvulsants,[138-140] pharmacokinetic studies of CBZ in dogs[141] and in humans,[142] kinetics of a CBZ-ethosuximide (33b) interaction,[143] and autoinduction of CBZ metabolism in children,[144] have been reported.

The pharmacological and EEG profiles for the benzisoxazole sulfonamide AD-810 (35) have been reported.[145,146] Another sulfonamide, a carbonic anhydrase inhibitor UK-15454 (36), also protected against MES in mice.[147] A QSAR study relating anticonvulsant activity, as measured by protection against MES, and physicochemical substituent constants in a series of benzenesulfonamides 37 has been published.[148]



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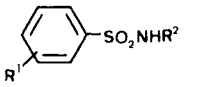
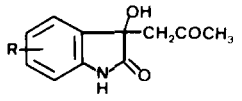


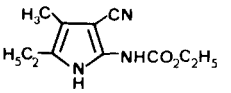
37, R_1 = p-CN, R_2 = C_2H_5

Other compounds reported effective in protecting against MES and/or

PTZ induced convulsions in rodents include oxindoles 38a,b,[149] substituted pyrrole 39,[150] 4-benzylidene-5-imidazolones 40a,b,[151] naphthoylmethylimidazole (NMI) 41,[152,153] and 1-aminocyclopentane carboxylic acid (42).[154]



38a, R = H
38b, R = 7-CH_3



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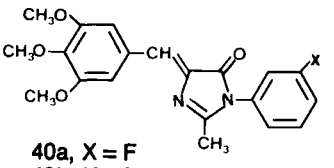
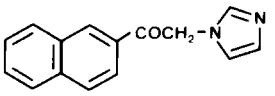


40a, X = F
40b, X = Br



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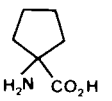

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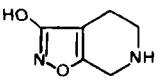

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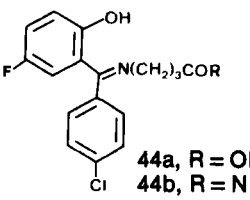
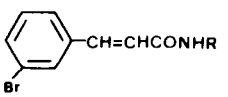
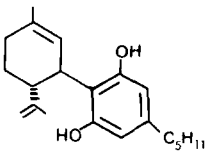


44a, R = ONa
44b, R = NH_2



45a, R = H
45b, R = C_2H_5



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While the GABA agonist THIP (43) was not effective against reflex epilepsy,[155] the GABA mimetics SL 75102 and SL 76002 (44a,b) were effective in antagonizing bicuculline-induced clonic convulsions in mice.[156] The effectiveness of 3-bromocinnamamide (45a), the major metabolite of the anticonvulsant cinromide (45b), in controlling seizures in epileptic monkeys has been reported.[157] A structural comparison of two anticonvulsants, cannabidiol (46) and phenytoin (33a), has been made utilizing conformational energy maps.[158] Both 1,2,3,4-tetrahydro-β-carboline (15c) and its 6-OCH_3 derivative 15d were effective in attenuating audiogenic seizures in DBA/2J mice.[159]

The decapeptide caerulein and the chemically related C-terminal octapeptide of cholecystokinin, CCK-8, have been shown to antagonize the toxic effects of the convulsants strychnine, PTZ, bicuculline and picrotoxin, suggesting that both may have anticonvulsive activity. It was suggested that the anticonvulsive effect of crude preparations of substance P may be largely attributable to CCK-like peptides.[160]

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Chapter 5. ANALGESICS (PERIPHERAL AND CENTRAL), ENDOGENOUS OPIOIDS AND THEIR RECEPTORS

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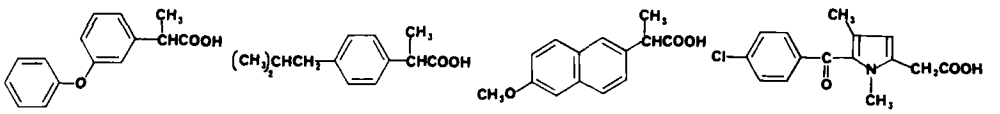
Introduction

By the end of 1980, four non-steroidal anti-inflammatory drugs had been introduced as peripherally acting oral analgesics. These agents have proven effective in a wide variety of pain situations formerly thought treatable only with narcotic (centrally acting) analgesics. Recent advances in the understanding of the mechanisms of analgesia, tolerance and physical dependence hold promise for the development of new and improved centrally acting analgesics. Research related to endogenous opioids continues to expand rapidly, and the syntheses of a variety of opioid peptides have been reported. The peptidases that inactivate enkephalins have been further purified and characterized, and potent specific inhibitors which produce analgesia have been developed. Efforts continue to characterize the opioid receptors. Evidence favors the existence of distinct, multiple opioid receptors, and the functions of the individual receptors are being sought. The search for specific agonists and antagonists of these receptors has intensified.

Clinical Studies

Peripherally Acting Analgesics - Recently, four orally effective non-steroidal anti-inflammatory drugs (NSAIDs), fenoprofen (Nalfon®), ibuprofen (Motrin®), naproxen (Anaprox®) and zomepirac (Zomax®), were approved by the Food and Drug Administration for the treatment of pain. These agents are not classified under the Controlled Substance Act; and, because they act in the periphery, they are free from many of the CNS depressant effects commonly associated with opiate-like analgesics.



Clinical testing has shown these agents to be more effective than aspirin, comparing favorably with codeine and the narcotic combination products, not only for the relief of arthritic pain but also for the relief of several types of postoperative pain. For example, 100 mg zomepirac was as effective as 60 mg codeine in the treatment of moderate to severe postoperative pain,[1] while 400 mg ibuprofen was significantly more effective than 65 mg propoxyphene hydrochloride in the relief of postepisiotomy pain.[2] In two separate studies, 100 mg oral zomepirac was significantly more effective than 8 mg intramuscular morphine in patients with moderate to severe postsurgical pain.[3,4] Additional studies with fenoprofen,[5] ibuprofen,[6] naproxen[7] and zomepirac[8,9] have been reported.

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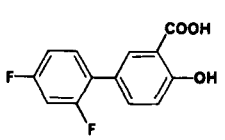
There were reports of other NSAIDs under clinical evaluation as peripheral analgesics. Diflunisal (Dolobid®) is related to salicylic acid and is reported to have greater potency and to be better tolerated than aspirin.[10] Clinical analgesic studies have also been reported with fluproquazone (RF46-790).[11] The antinociceptive properties of suprofen have been described along with data suggesting it may be a tissue-selective inhibitor of cyclooxygenase activity.[12] Oxapadol is described as a new non-narcotic analgesic and has shown analgesic activity in a human experimental pain model.[13,14]



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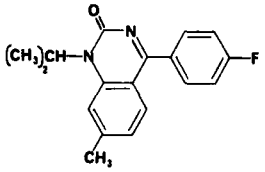

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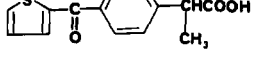

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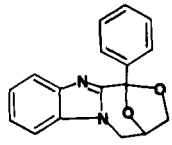


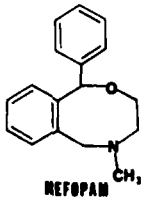
OXAPADOL

The mechanism by which NSAIDs relieve pain has been recently reviewed.[15-18] It is generally accepted that prostaglandins, released by tissue injury, sensitize nociceptors and that NSAIDs produce analgesia by prevention of this sensitization through the inhibition of prostaglandin synthesis. NSAIDs are thought to exert their effects peripherally; however, there are reports that these agents may also act centrally.[18,19]

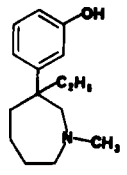
Because of their different mechanisms of action, a peripherally or centrally acting analgesic may have different levels of effectiveness in various clinical situations. Studies to explore the differential effects of these agents have been reported.[20-22] The use of NSAIDs represents an important new therapy for primary dysmenorrhea.[23]

Centrally Acting Analgesics - Of the second generation mixed agonist-antagonists, only the injectable forms of butorphanol (Stadol®) and nalbuphine (Nubane®) have been marketed in the United States, while buprenorphine (Temgesic®) has been marketed in the United Kingdom. Several studies have shown these agents to be safe and effective for the relief of moderate to severe pain, and they appear to have a low physical dependence and abuse liability. Butorphanol is also effective following oral administration; 8 mg is at least equivalent to 60 mg codeine.[24,25]

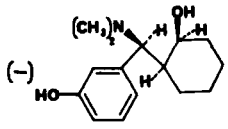
The clinical evaluation of nefopam (Acupan®), a non-narcotic centrally acting analgesic continues. Its pharmacological properties and therapeutic efficacy have been reviewed.[26]



NEFOPAM



MEPTAZINOL



CIRAMADOL

Meptazinol, a novel mixed opiate agonist-antagonist, is being clinically evaluated for both parenteral and oral usage. In postoperative pain patients, meptazinol (75 and 100 mg, i.m.) was equi-analgesic with meperidine (100 mg, i.m.) with a similar frequency of side effects.[27]

Ciramadol (Wy15,705) is a benzylamine derivative with mixed opiate agonist-antagonist properties.[28] In patients with chronic cancer pain, ciramadol at 20 mg and 60 mg, i.m., produced a dose-related analgesic effect.[29] No side effects were observed.

There were clinical reports of two enkephalin analogs. FK33-824 (1 mg, i.m.) was found to produce effects similar to morphine against electrically induced pain in humans.[30] In another study, FK33-824 (1.25 mg, i.m.) was found to reduce withdrawal symptoms in chronic heroin abusers but was less effective than morphine. Side effects with FK33-824 included a feeling of oppression in the chest and heaviness in muscles. Some subjects refused to take a second dose.[31]

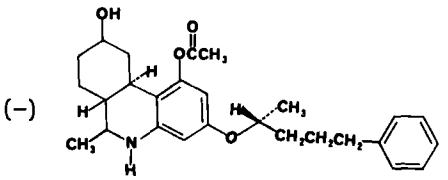
H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol

FK33-824

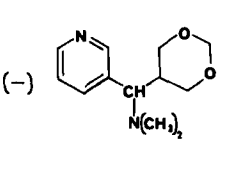
H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2

Metkephamid

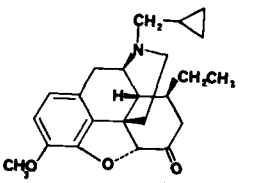
Metkephamid (LY127623) is a systemically active analog of methionine enkephalin (Met-enk).[32] In rodents, metkephamid (s.c.) has an analgesic potency comparable to that of meperidine and produces less respiratory depression and physical dependence than morphine. In a Phase I clinical study, metkephamid (0.5 to 90 mg, i.m.) caused nasal congestion, dry mouth, heaviness of limbs and changes in emotional state.[33]



LEVONANTRADOL



DOXPICOMINE



CODORPHONE

Levonantradol is a synthetic cannabinoid derivative currently under clinical evaluation for its analgesic properties. In rodents, it has an analgesic potency 2-7 times that of morphine. The analgesic effects of levonantradol are not mediated through opiate receptors, but rather, levonantradol appears to interact with a novel and as yet undefined receptor.[34] Doxpicomine (formerly doxpicodin) is a novel partial opiate agonist that was shown to produce an analgesic effect in rodents comparable to that of meperidine and codeine, with a wide margin of safety.[35] It has also demonstrated low physical dependence liability and limited respiratory depression. Clinical studies are in progress. Codorphone is a mixed opiate agonist-antagonist undergoing Phase I clinical trials.[36] The SAR of the series of compounds related to codorphone has been published.[37,38]

Opioid Peptides

Recent reviews relating to the endogenous opioids describe the current status of opioid peptides,[39-42] opiate receptors[43,44] and SARs of enkephalin analogs.[45,46] Other review articles and lead references to areas not covered in this chapter discuss opioid peptide precursors;[47-49] distribution of Met-enk, leucine enkephalin (Leu-enk)[50] and endorphins;[51] conformation studies;[52-54] behavioral aspects;[55] neuroendocrine effects;[56] appetite control;[57] and the relation of opioid peptides to schizophrenia,[58] shock[59,60] and respiratory depression.[61] Finally, reviews of peptide receptors in the brain,[62] opioid and other peptides as neurotransmitters,[63-65] the transmembrane transport of small peptides[66] and

design and development of biologically active peptides as drugs[67,68] have been published.

Enkephalin Analogs - Several SAR studies were reported during 1980.[69,70] Modification of the amide bond between the Tyr and Gly in an enkephalin by substitution with either a trans carbon-carbon double bond,[71,72] a $-CH_2-CH_2-$, or a $-CH_2-NH-$ moiety,[73] gave analogs that retained significant biological activity. Several novel amino acid substitutions have been reported. The type and location of these substitutions are as follows: [guanidino-Tyr^1];[74] [D,L-β-ferrocenylalanine^4];[75] [L-t-butylglycine^5];[76] and [aminosulfonic acid^5] and [aminophosphonic acid^5].[77] Several of these analogs were highly resistant to peptidase degradation.[76,77] To date, no structural analog of enkephalin displaying narcotic antagonist properties has been reported.

Other Peptides - Dynorphin (DYN), formerly the "slow reversing endorphin," contains 17 amino acid residues.[78] The structure of the C-terminal tetrapeptide fragment is unreported;[79] however, the N-terminal fragment, DYN (1-13), retains full biological activity. Several synthetic analogs of DYN (1-13) have been described.[79,80] Although DYN has been suggested to be a possible Leu-enk precursor, the regional distribution of DYN in rat, bovine and porcine brain and pituitary does not parallel that of any known opioid peptide.[79]

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH DYN (1-13)

Opiates (i.v.) characteristically reduce blood pressure and heart rate. Whereas β-casomorphin did not display appreciable cardiovascular activity, the tetrapeptide fragment (H-Tyr-Pro-Phe-Pro-NH_2) was equipotent with the enkephalin analog [D-Ala^2,D-Leu^5]-enkephalin (DADL) in producing bradycardia.[81] All possible amino-terminal fragments of β-casomorphin have been synthesized and studied in the guinea pig ileum (GPI) assay. The most potent fragment (H-Tyr-Pro-Phe-Pro-Gly-OH) possessed an IC_{50} of 6.5 μM.[82]

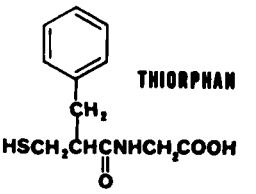
H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH β-casomorphin

Further evidence has been presented that substance P is the neurotransmitter released by primary nociceptive afferents.[83] The analgesic activities of substance P and its analogs have been described.[84-86] The mechanism by which substance P produces analgesia has been suggested to be inhibition of enkephalin degradation.[87] It has been reported that morphine acts presynaptically, rather than as a substance P antagonist.[88] Since substance P does not bind to opiate receptors,[87] substance P antagonists could represent a novel class of analgesic agents potentially free from opiate-like side effects.

Enkephalinase - The peptidases that degrade and inactivate the enkephalins have been further purified and characterized. The dipeptidylcarboxypeptidase (enkephalinase A) has been demonstrated to be distinct from angiotensin converting enzyme (ACE).[89,90] Enkephalinase A activity has been dissociated into two distinct dipeptidylpeptidases, which have been designated enkephalinase A_1 and A_2. Enkephalinase A_1 has been purified to homogeneity.[91] A soluble enzyme, which cleaves the Tyr-Gly bond of Met- and Leu-enk with high specificity, has been isolated from human brain and purified to homogeneity. This enzyme, ML-enkephalinase, displayed a number of characteristics similar to those of arylamidases and was suggested to play a role in physiological CNS-enkephalin homeostasis in man.[92]

Enkephalinase Inhibitors - The search for potent and specific inhibitors of the enkephalin-degrading enzymes represents a new direction in the search for analgesic agents. Recent studies have centered upon the ability of captopril to potentiate morphine-[93] and Met-enk-induced[94] analgesia via inhibition of Met-enk metabolism. Although some analgesic potentiation was observed, interpretation was complicated by influences on the kallikrein-kinin system.[93] The inhibition of ACE by β-endorphin (β-EP), Met-enk and Leu-enk has recently been reported. Thus, the opioid peptides may influence the angiotensin I-bradykinin system and, therefore, participate in cardiovascular control.[95]

Initial results with the rational design of specific enkephalinase inhibitors are encouraging. Enkephalinases A_1, A_2 and B (dipeptidyl-aminopeptidase) and aminopeptidase are metallo (Zn) enzymes[91] that can be inhibited by metal ion chelators, such as puromycin or o-phenanthroline.[89,91,96] Based on this fact, a series of protected amino acid hydroxamates (transition metal chelators) has been synthesized. The most potent analog (Z-Leu-NHOH) possessed a K_I of 40 nM against enkephalinase A.[97]



Careful examination of the substrate specificity of enkephalinase A from mouse striatum has led to the development of a potent and specific enkephalinase A inhibitor, thiorphan, with a K_I of 4.7 nM.[98] The in vivo pharmacology of thiorphan is complex. In the tail-withdrawal test, thiorphan (systemic or i.c.v.) strongly potentiated the analgesic effect of i.c.v. administered [D-Ala^2]-Met-enk; however, it did not produce an analgesic response by itself. In the mouse hotplate-jump test, however, thiorphan alone (i.c.v. or i.v.) produced naloxone-reversible analgesia.[99] Further results with these and other synthetic enkephalin peptidase inhibitors are eagerly awaited.

Blood Brain Barrier - Several studies have been published which specifically address the controversy of the entry of opioid peptides, especially β-EP, into the CNS; however, no definitive conclusions have been reached.[100,101] The epidural and intrathecal (injection directly into the spinal CSF) routes of administration are gaining popularity as methods which bypass the blood-brain barrier.[102] This technique has been successfully used with morphine[103] and β-EP during labor.[104] Intrathecal administration of β-EP in cancer patients with intractable pain was reported to give rapid, profound and long-lasting analgesia (mean duration of pain relief was 33.4 hours).[105]

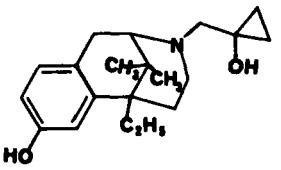
Opioid Receptors

Classification - Available evidence favors the hypothesis of multiple opiate receptors. Unfortunately, the nomenclature is confusing since various opiate receptors have been referred to as μ, σ, κ, δ, ε, type I, type II, high affinity, low affinity, O_1, or O_2! The pharmacological evidence is now compelling for at least two distinct receptors designated μ (morphine) and δ (enkephalin). Extensive competitive binding assays in guinea pig brain homogenates,[106] combined with comparative pharmacology in the GPI assay and the mouse vas deferens (MVD) assay,[107] have been reported, which strengthen the case for separate δ- and μ-receptors. The opiate binding in homogenates of rat brain can be irreversibly inactivated by N-ethylmaleimide (NEM). The presence of opiates or enkephalins during NEM incubation resulted in protection of μ- or δ-receptors, respectively.[108]

Similar results were reported earlier using phenoxybenzamine as an irreversible inhibitor of opiate receptor binding.[109] Traditionally, opioid effects in GPI and MVD have been correlated with binding to μ- and δ-receptors, respectively. Now enkephalin binding sites (δ-receptors) have been described in rat, guinea pig and rabbit ileum.[110] Chronic treatment of mice with DADL rendered their vas deferentia insensitive to δ-receptor ligands, such as DADL, but not to μ-agonists. Conversely, after infusion with the potent μ-agonist sufentanyl (SUF), their vas deferentia were insensitive to μ-receptor ligands but responded normally to DADL.[111] Thus, mouse vas deferentia contain μ- as well as δ-receptors. Neuroblastoma X glioma hybrid cells have been reported to possess only δ-receptors.[112] The peptide H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH has been reported to be 5-6 times more selective for the δ-receptor than DADL,[113] which was previously the most selective ligand available for this receptor.

If mice are infused simultaneously with DADL and SUF, their vas deferentia are insensitive to all opioids investigated, except DYN (1-13). This finding has been explained by the presence of highly selective DYN receptors in the MVD.[114] The term ε-receptor has been proposed for the opiate receptor in the rat vas deferens which has pronounced selectivity for β-EP as ligand.[111,115]

It has been suggested that bremazocine is a potent and long-acting opiate κ-agonist.[116] However, the evidence for κ-receptors is not as definitive as for μ- and δ-receptors. Studies with the κ-agonist [^{3}H]-ethylketocyclazocine have failed to support the existence of a separate κ-receptor in rat brain membranes.[117,118]



BREMAZOCINE

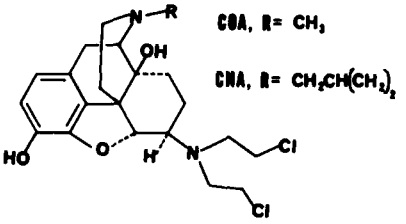
Research efforts are now beginning to focus on the specific responses mediated by each opiate receptor. Several reports suggest that μ- and/or κ-receptors are the mediators of analgesia, while δ-receptors mediate other effects.[117,119-121] The "low affinity" receptor has been suggested to mediate respiratory effects, while the "high affinity" receptor mediates analgesia.[122]

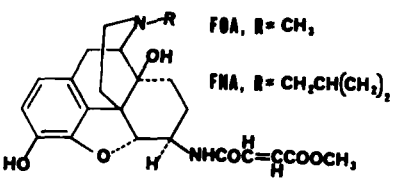
It has been reported that opiate agonists and antagonists will induce opiate receptors to form sulfhydryl-disulfide dependent clusters.[123] Interestingly, cupric ion, which is a thiol oxidant, has been shown to elicit naloxone-reversible analgesia (i.c.v.), with potency equivalent to that of morphine.[124]

Several membrane complexes assumed to be portions of opioid receptors have been isolated from mouse brain by use of an irreversible opiate antagonist.[125] A soluble opiate receptor preparation with stereospecific binding properties has been isolated from rat neural membranes.[126]

Irreversible Opioid Agonists and Antagonists - The pharmacological effects of the irreversible opiate agonist chloroxymorphamine (COA) and antagonist chlornaltrexamine (CNA) were reported.[127] Intracerebroventricular injection of CNA was found to antagonize morphine-induced analgesia for three days, while administration of COA produced prolonged analgesia that could be blocked and reversed with naloxone. It was shown that naloxone reversal of the analgesic effect of COA was not due to displacement of COA from opioid receptors, suggesting the existence of

separate agonist and antagonist binding sites. The synthesis of another pair of opioid receptor probes, FOA and FNA, has been reported.[128] Data were presented that indicated only FNA binds irreversibly to opioid receptors even though both FOA and FNA formed receptor complexes. These results further support the existence of multiple receptors or multiple binding modes of a single receptor.





Naloxazone, a hydrazone derivative of naloxone, has been shown to be a long-acting opioid antagonist, both by in vivo and in vitro measures. Interestingly, in mice, naloxazone blocks the analgesic activity without affecting the LD_{50} of morphine.[129]

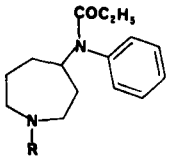
There were reports on the preparation of opioid receptor labels using photosensitive arylazide enkephalin analogs.[130,131]

Opiate Dependence and Tolerance - The evidence that opiate dependence and tolerance develop within neurons containing specific opiate receptors has been reviewed.[132] The molecular mechanism of opiate dependence appears to be related to the inhibition of adenylate cyclase.

Additional studies concerning the mechanisms of opiate dependence and tolerance were reported. By using the (+) and (-) isomers of naloxone, an opiate-withdrawal response was demonstrated on single neurons in the morphine-dependent rat brain.[133] The oxytocin-derived fragments H-Pro-Leu-Gly-NH_2 and cyclo(Leu-Gly) block the development of physical dependence and tolerance to morphine, without affecting its analgesic response.[134] Development of tolerance to opiates was suggested to be associated with enhanced dopamine receptor sensitivity.[135] Further evidence that enkephalin analogs will produce or support physical dependence has been presented.[136]

Chronic administration of opiate agonists and antagonists has failed to alter Met-enk and β-EP levels in areas of rat brain and pituitary.[137] However, changes in β-EP fragments were found after chronic morphine treatment of GPI.[138]

New SAR Studies - The syntheses and analgesic activities of series 1,[139] 2 [140] and 3[141] were reported this year. In the 4-amino-4-arylcyclohexanones (4), the effects of aryl substitution were explored.[142]



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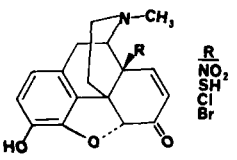

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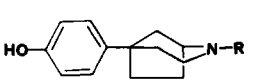

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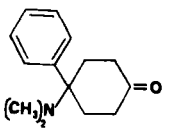

(4)

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Chapter 6. Memory and Learning

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Introduction - Effects of drugs on memory and learning are extremely difficult to assess in animals or man. Animals can be trained to perform certain tasks in order to obtain rewards or to avoid punishment, and the ability to learn these tasks (acquisition) or to continue performing them even when the rewards or punishment are absent (extinction) can be influenced by treatment with drugs. Drug-induced changes in acquisition or extinction are interpreted to indicate that the drug has affected memory processes.[1,2] However, the effectiveness of drugs that impair (retrograde amnesia) or facilitate (delay extinction) retention is sensitive to dose, type and degree of training, and time of drug administration. Thus, problems with the reproducibility of data are common. In view of these difficulties, it is perhaps not surprising that only one drug, Hydergine™, has been approved for marketing in the U.S. and Europe for treatment of disorders that involve memory deficit in man.[3]

The recent trend has been to combine behavioral results with biochemical measurements in an attempt to identify brain structures and systems that may be involved in the memory process.[4,5] Many studies that have focused on measuring the consequences of electrical stimulation or microinjection of drugs into specific brain loci have shown that behaviorally-active drugs affect neurotransmitter systems,[6] particularly noradrenergic[7] and dopaminergic[4] pathways. The concentrations of phosphoprotein and glycoprotein in rat brain also are changed by training.[4] Although the functional roles for synaptic transmission and macromolecular synthesis[8] in the memory process are not clear, the increased use of biochemistry and the evolution of a cellular model for memory formation[9] suggest that progress is being made. Although there are considerable data to support a role for noradrenergic[10-17] and GABAergic systems[18-24] in memory processes, this review will concentrate on vasopressin and cholinergic-type drugs where some clinical experience exists which ultimately may be useful in determining whether or not the current experimental approaches to discovering memory-enhancing agents are valid. The presence of a selective dysfunction in central cholinergic mechanisms in Alzheimer's disease,[25] which is characterized clinically by memory and learning deficits, makes the study of cholinergic agents particularly relevant. Vasopressin and other pituitary peptides are of interest because they may modulate noradrenergic pathways which have been implicated in the memory process.

Compounds Affecting the Cholinergic System - Studies in rodents have shown that administration of drugs that block the receptors for acetylcholine (ACh), such as scopolamine, atropine and hyoscine, can impair memory and cognition. Conversely, drugs that stimulate ACh receptors either directly (e.g., oxotremorine, arecoline) or indirectly by inhibition of acetylcholinesterase (AChE) (e.g., physostigmine) either overcome the impairment of memory caused by receptor blockers or can, by them-

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selves enhance performance.[26-39] Deutsch and his coworkers[40-42] have advanced the hypothesis that modifications of the cholinergic synapse form the physiological basis of memory. The theory is based upon the finding that cholinergic agonists will either block or restore memory in rats, depending upon the time between training and retesting the animals (i.e., the "age" of memory), whereas cholinergic receptor blockers produce effects that are the exact opposite of cholinergic agonists. The "strength" of memory also determines whether or not a cholinergic agonist will produce improvement; a poorly-trained task can be facilitated, whereas a well-trained task may be blocked by cholinergic stimulants.[41]

Studies in humans tend to corroborate the data in rodents. For example, administration of scopolamine to young students impaired memory storage and retrieval, as well as other cognitive functions.[43] This finding essentially agrees with other results in humans[44-46] and with data from monkeys.[47] The impairment of cognitive function in both humans[43] and primates[47] was similar to that found in normal aged subjects. In these and other studies, the deleterious effect of scopolamine on performance of normal subjects was reversed by administration of physostigmine[44,48,49] or arecoline[50] but not by amphetamine at a dose that improves alertness.[48] (In another study, methamphetamine was found to antagonize the memory impairment induced by scopolamine in humans.[51]) Moreover, the inhibition of performance induced by diazepam, which does not block cholinergic receptors, was not reversed by physostigmine.[49] These results argue for a specific involvement of cholinergic neurons in the behavioral deficit induced by administration of scopolamine.

Neurochemical data, showing a loss of central cholinergic neurons as deficits in memory during aging, also tend to support the pharmacoligic evidence from humans and animals for a specific role of the central cholinergic nervous system in memory and learning. The best neurochemical marker for cholinergic neurons is choline acetyltransferase (ChAt), which is present exclusively in cholinergic neurons. It is well established that the activity of ChAt declines in brain as animals grow older. In a group of 28 human patients that had died from non-neurological causes, ChAt was found to decrease from 40% to 66% in cortical areas as the age of the patients increased from 20 to 50 years.[52] The decline in ChAt activity was especially prominent in the septal area, which supplies neurons to the hippocampus, a region of the brain that is prominently associated with memory consolidation. In patients with Alzheimer's-type senile dementia, who are afflicted with memory loss that is similar to, but more severe than, that of normal aged people, the loss of ChAt activity is even more pronounced than in age-matched controls, and is most pronounced in the amygdala, hippocampus, and cortex.[53-56] Furthermore, the decrease in ChAt that occurs in brains of Alzheimer's patients is correlated with the degree of neuronal degeneration and cognitive loss that occurs in aged patients.[57] Markers for other types of neurons (e.g., GAD for GABA; tyrosine hydroxylase for catecholamines) are not severely affected in Alzheimer's patients,[55] suggesting a selective loss of cholinergic neurons in this disease. While nearly unanimous agreement exists concerning loss of ChAt with age, evidence for changes in muscarinic receptors is controversial. In aged rats,[58] normal aged humans[56] and Alzheimer's victims,[59] a decrease in receptors was observed, whereas in other studies of patients with senile dementia no such changes had occurred.[56,60]

TABLE I. SUMMARY OF CLINICAL STUDIES WITH CHOLINERGIC AGENTS

Treatment	Subjects	Effect	Reference
Physostigmine			
1.0 mg., S.C.	?	Improved	44
0.6 mg., S.C.	8, Y	Improved	62
0.3 mg., I.V.	13, Y	Worsened	64
0.8 mg., S.C.	1, Amnesia	Improved	63
1.0 mg., I.V.	19, Y	Improved	65
0.5 mg., I.V.	6, E	Improved	66
0.02 mg., I.V.	6, Y	Improved	67
1.0 mg., S.C.	1, A	Improved	68
1.0 mg., S.C.	1, A	Improved	69
0.8 mg., S.C.	13, E	Improved	70
Arecoline			
4.0 mg., S.C.	14, Y	Improved	50
Choline[a]			
10 X 1	10, Y	Improved	85
10 X 14	5, A	Improved	86
9 X 14	10, A	No Effect	87
10 X 7	8, E	No Effect	88
16 X 3	15, Y	No Effect	89
8 X 3	9, Y	No Effect	90
8 X 20	10, E	No Effect	91
Lecithin			
?	5, A	Improved	92
Deanol[a]			
0.5 X 90	74, MBD	Improved	94
0.9 X 21	11, E	No Effect	95
Meclofenoxate[a,b]			
1.2 X 63	28, E	No Effect	97
1.2 X 180	76, E	Improved	98
1.5 X 28	16, Y	Improved	98

[a] Doses are grams/day X number of days (P.O.).
[b] Ester of deanol that is hydrolyzed *in vivo*.

A = Alzheimer's; Y = Young normals; E = Elderly;
MBD = Children with minimal brain dysfunction.

Cholinergic Therapy for Memory and Learning Disorders - Clinical investigators have used cholinergic agonists to treat humans suffering from memory disorders. Physostigmine has been the prototype cholinomimetic compound for most human studies, although a report by Bartus[61] using rhesus monkeys suggests that this compound may not be the best. Bartus found that performance of young rhesus monkeys was improved by physostigmine and that the dose-response curve was a bell-shaped function that was fairly uniform between animals, with improvement occurring only within a very narrow range of doses. However, in aged monkeys, the dose-response curve varied considerably between individual monkeys such that some monkeys improved with extremely low doses of physostigmine, whereas others were improved by doses that had impaired the performance of younger monkeys.

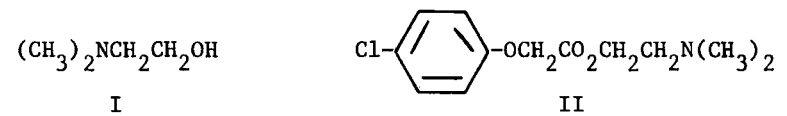
Despite the questionable validity of negative clinical results in which a single dose of a cholinomimetic agent was studied, several

investigators have found that administration of cholinergic drugs such as physostigmine improves memory.[62-70] These results, summarized in Table I, tend to suggest that lower doses of physostigmine are more effective than higher doses, and that improvement is more likely to occur when memory is impaired. Direct-acting cholinomimetics have not been extensively studied in humans, even though they have been shown to improve memory in mice.[71] A clinical study with arecoline demonstrated significant improvement in the rate of serial learning of familiar words.[50] Moreover, the compound was more effective in poor performers than in subjects who learned more rapidly.

Choline and its precursor, lecithin, have been tested for effects on learning because administration of choline to animals seems to stimulate the synthesis and release of ACh.[72] Choline added to the diets of old mice improved their learning ability as compared to young mice given a choline-deficient diet.[73] When given together with piracetam, an agent that improved performance in some[74-80] but not all[81-83] animal or human experiments, choline improved the memory performance of aged rats, whereas either compound administered alone was without effect in these animals.[84]

Despite these findings in rodents, clinical studies of the effects of choline and lecithin on memory do not look promising. As shown in Table I, of the 8 studies reported, choline was effective in one group of normal volunteers given a single dose[85] and in 5 Alzheimer's patients treated for 2 weeks,[86] but not in the 6 other studies.[87-91] Because of the fishy odor produced when high doses of choline are administered, lecithin is now being used. In one published study, the ester was found to be effective in 5 Alzheimer's patients, but only when given with physostigmine.[92] In this study, physostigmine by itself had no effect on memory. Although additional studies with lecithin are underway, it seems unlikely that either choline or lecithin when given alone will be as effective in the treatment of impaired learning, as they have been in the treatment of movement disorders such as tardive dyskinesia.

Deanol (I), a compound structurally related to choline that causes an increase in choline and ACh in brains when given to rats,[93] also has been used successfully to treat movement disorders. Treatment with deanol improved learning in children with minimal brain dysfunction,[94] but did not improve cognitive performance in elderly humans.[95] An ester of deanol, meclofenoxate (II), improved memory in aged mice, and reduced the content of neuronal lipofuscin pigment, which is characteristically high in aged neurons.[96] Meclofenoxate also has improved memory in several clinical trials (see Table I).[97,98]

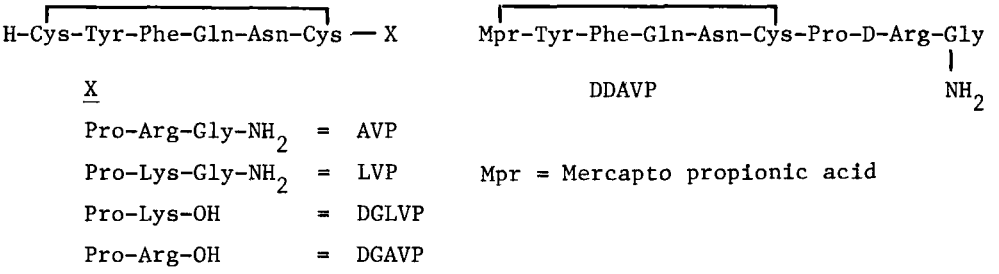


I II

In conclusion, experiments performed using subhuman species overwhelmingly suggest that cholinomimetic drugs should improve memory and cognition in humans who do not perform well, and this view is supported by many favorable clinical reports such as those summarized in Table I. The major disadvantages of cholinomimetic compounds as therapeutic agents are (1) their short duration of action and (2) the unpleasant peripheral side effects (e.g., diarrhea, salivation) which can only be

overcome by simultaneous administration of a peripheral antimuscarinic agent. Although many investigators had hoped that choline (or lecithin) would overcome both problems, the precursor of ACh does not seem to be as effective as the direct cholinergic receptor stimulants (e.g., physostigmine) in old people, possibly because of the loss of cholinergic nerve terminals (and ChAt) needed to synthesize the neurotransmitter. A few studies suggest that combination therapy (e.g., choline plus piracetam; lecithin plus physostigmine) may improve performance.

Vasopressin - The recent focus for studies on the effects of peptide hormones in memory processes has shifted from the shorter-acting $ACTH_{4-10}$ analogs to vasopressin, which has a longer duration of effect in facilitating acquisition and delaying extinction of avoidance responding in rats.[99-102] Thus, the extinction of pole-jumping avoidance responding in rats is delayed for days by post-training administration of a single dose of [Arg^8]vasopressin (AVP), [Lys^8]vasopressin (LVP), or [des-GlyNH_2^9]LVP (DG-LVP), which is devoid of typical endocrine effects.[103,104] In passive avoidance paradigms,[105,106] the response latency of mice and rats is increased when vasopressin is given immediately after trial or 1 hour before a subsequent retrieval test. These results suggest that both consolidation and retrieval processes are influenced by treatment with this peptide.[102] Experiments with a retrograde amnesia design also can be used to detect the apparent memory facilitating effect of vasopressin. Retrograde amnesia for a passive avoidance task induced in rats by electroshock,[107] CO_2 inhalation, or treatment with puromycin[108] or pentylenetetrazol[109] is antagonized by vasopressin given just before training or prior to retrieval testing. Amnesia induced by the dopamine-β-hydroxylase inhibitor, diethyldithiocarbamate (DDC), also is inhibited by vasopressin but only when given prior to retrieval testing.[110] While vasopressin has anti-amnesic activity, the structurally-related neurohypophyseal hormone oxytocin is an amnesic peptide.[105] In behavioral and biochemical assays, its effect is opposite to that of vasopressin.



It has been shown that rats which are deficient in vasopressin have deficits in acquiring and maintaining avoidance behavior which can be corrected by treatment with this peptide.[102,111] Administration of vasopressin also restored normal hippocampal theta rhythm in these rats.[112] These results suggested that the facilitating effect of vasopressin on avoidance behavior is physiologically significant and prompted further studies to identify the site(s) and mechanism of action through which this effect is expressed.[113-117] The results suggested that vasopressin affects memory processes through an action on the terminals of the dorsal noradrenergic pathway. Thus, microinjection of vasopressin into specific limbic mid-brain structures which contain projections of the ascending noradrenergic bundle delayed extinction of active avoidance

behavior, increased passive avoidance latency, and increased the turnover rate of norepinephrine.[113,114] Microinjection of antivasopressin serum at these sites had the opposite effect.[115,116] No effect on these parameters was observed when the peptide or its antiserum was injected into the locus coeruleus (noradrenergic cell body region for the dorsal pathway). Furthermore, destruction of the dorsal noradrenergic bundle[118] with 6-OHDA or pre-treatment with a catecholamine synthesis inhibitor prevented the facilitating effect of vasopressin on passive avoidance behavior.[114] While specific limbic structures appear to be important for the expression of this effect of vasopressin on avoidance responding, it appears that the limbic system need not be intact, because transection of the fornix does not prevent the delay of extinction in active avoidance responding caused by the behaviorally active vasopressin analog DG-LVP.[119] The extrahypothalamic existence and distribution of vasopressin[120,121] and vasopressinergic fibers[122,123] in rat brain have been demonstrated. Thus, modulation of catecholamine neurotransmission via vasopressin receptors[124] on noradrenergic neurons may contribute to the behavioral effect of this peptide.

Interactions between opiate peptide systems and vasopressin pathways also may be of functional significance in memory processes. It has been demonstrated by radioimmunoassay that β-endorphin, which has an amnesic effect when administered immediately after training in an avoidance paradigm, is released endogenously in the rat during training.[125] This peptide, as well as morphine and the stable D-Ala^2, D-Leu^5-enkephalin (DADLE), has been shown to inhibit the electrically-stimulated release of vasopressin in vitro from rat pituitary.[126] The inhibitory effect of DADLE can be reversed by the opiate antagonist naloxone, which has been found to delay extinction of avoidance behavior in the rat.[127-129] Furthermore, morphine and β-endorphin decreased plasma vasopressin levels,[130] and morphine inhibited the firing of vasopressinergic neurons in the rat by a naloxone reversible process.[131] While endogenous β-endorphin is released during training in an avoidance paradigm, it is not released during retrieval testing.[125] When β-endorphin or the enkephalins were administered 1 hour prior to the retrieval test, the extinction of the avoidance response was delayed.[132,133] These observations suggest, but do not establish, a role for opiate peptide-neurohypophyseal hormone interactions in the memory process.

Treatment with LVP nasal spray has been reported to correct memory deficits in patients with post-traumatic amnesia,[134] improve memory in patients with Korsakoff syndrome,[135] and enhance performance by elderly volunteers in tests involving attention, concentration and memory.[136] However, in other studies,[137,138] intranasal treatment with LVP, 1-desamino-8-d-arginine vasopressin (DDAVP), an AVP analog with prolonged half-life, high diuretic and low pressor activity, and DG-AVP was without benefit to patients with memory deficit resulting from head injury. LVP did not improve memory in patients with Parkinson's disease.[139] Contrary to the above results, DDAVP was found in two separate studies[140,141] to improve cognitive function in patients with major affective illness, as well as in unimpaired volunteers. Although DDAVP corrected a deficiency for learning a passive avoidance response, it did not prevent self-mutilation activity in a Lesch-Nyhan child.[142] Intranasal or intravenous administration of a large dose of oxytocin to six volunteers did not appear to affect learning but did impair recall, suggesting that the peptide impaired cognitive function.[143] More extensive trials will be needed to establish utility for these peptides in treating certain

deficits in cognitive function.

ACTH(4-10) and Analogs - The rate of acquisition of avoidance behavior in rats is increased by ACTH(4-10) and related analogs which lack endocrine action. The extinction of previously acquired behavior (conditioned taste aversion, food-rewarding T-maze, conditioned shock avoidance and pole-jumping avoidance) is delayed by these peptides. These results have been interpreted in terms of improved motivation and attention. Clinical results have supported this contention.[144] Recent clinical results are in accord with earlier work. Thus, studies with ACTH(4-10) and [Met$(O_2)^4$,D-Lys^8,Phe^9]ACTH(4-9) (Org 2766) demonstrated reduction in duration of lapses and number of errors by volunteers in a continuous performance task.[145,146] Org 2766 is orally active and behaviorally is one thousand times more potent than ACTH(4-10). In another trial, ACTH(4-10) improved visual attention in a group of alert, elderly (62-85 years) volunteers.[147] In mentally-retarded adults, Org 2766 improved attentional processes.[148] In volunteers with mild senile organic brain syndrome, ACTH(4-10) reduced depression and confusion and improved vigor in neuropsychological tests.[149] Org 2766 does not affect sleep, which distinguishes this compound from stimulants such as amphetamine and caffeine, which affect attention and sleep.[150]

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Section II - Pharmacodynamic Agents

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Chapter 7. Pulmonary and Antiallergy Drugs

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Introduction - A recent symposium[1] has provided a comprehensive survey of the current methods utilized in the evaluation and development of anti-allergic agents. The primary obstacle remains in the inability to correlate the preclinical assessment of a new drug with clinical efficacy. This is especially apparent in the case of mediator release inhibitors. Numerous cromoglycate (DSCG)-like compounds have been described (q.v.) that are readily absorbed orally and very effective in animal models, such as passive cutaneous anaphylaxis (PCA). However, they have been disappointing in bronchoprovocation studies in man.[2] The tendency has been to conclude that there is some special property in DSCG that is absent in the newer substances.[2] What has generally been ignored is the fact that the effect of a drug distributed systemically is compared with that of another (DSCG) applied directly to the bronchial mucosa. It is noteworthy that the efficacy of β-agonists in exercise-induced asthma is only realized by inhalation and not by oral administration.[3] Considering the complex etiology of asthma and the numerous receptors implicated, it is natural to inquire as to whether this observation may reflect a fundamental problem in drug distribution. Optimism, nevertheless, prevails with the development of more meaningful models[4] and a fuller awareness of the limitations of those in general use.[1]

Reviews have appeared on bronchial hyperreactivity,[5] lung mucus production and clearance,[6] the drugs currently in use in the management of allergic disorders[7] and the development and present status of the leukotrienes.[8] (For a comprehensive review of the latter subject, see Chapter 20).

Immunotherapy - A review of the current status of immunotherapy in asthma has appeared.[9] The importance of standardization of allergen extracts has been stressed.[10] The potential of specific and non-specific IgE suppression has received considerable attention.[11] Serum factors which modulate IgE production have been identified in mice[11] and rats.[12] Antigen exposure coincident with an imbalance in IgE suppressive/enhancing components has been suggested to be precursory to the development of the allergic state.[11]

General - The isolation of IgE receptors on rat basophils and mast cells and their role in the degranulation process has been reviewed.[13] The nature and function of the components of the IgE receptor[14] and the various types[15] identified on rat basophilic leukemia and mast cells are active areas of research. An excellent review[16] on granule fusion and exocytosis in rat peritoneal mast cells is recommended for reading as is a definitive study on IgE-mediated degranulation of human basophils[17] and a commentary[18] on the role of mast cells in allergic diseases. The cross-linkage of IgE receptors has been suggested to be the event that activates

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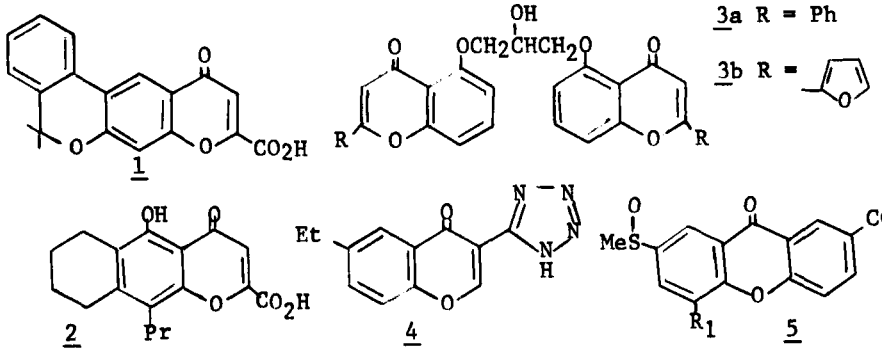
phospholipid methyl transferases I and II, which alter membrane fluidity and permit Ca^{++} influx.[19] It is also suggested that the subsequent activation of phospholipase A_2 provides arachidonic acid and lysophospolipids which promote membrane fusion. A recent review[20] on the function of the enzymes identified within the mast cell granule is noteworthy.



Inhibitors of Mediator Release - The influence of disodium cromoglycate (DSCG) in immunological and non-immunological processes has been reviewed.[21] The precise mode of action is as yet unresolved but does not involve inhibition of the influx of extracellular Ca^{++}.[22] That the site of action is at the membrane surface and not intracellular has been demonstrated with DSCG covalently bound to fluorescent polymer beads.[23] A preferred inhibition of lymphocyte cAMP phosphodiesterase (PDE) over that of mast cells has also been described.[24]

At high concentrations ($>10^{-3}$M), DSCG blocked mediator-induced contractions of guinea pig ileum.[25] A similar *in vitro* activity was reported with PRD-92 (1), but no effect was observed[26] in rats on intradermal serotonin or bradykinin at levels of 1 which inhibited the PCA reaction (30mg/kg p.o.).

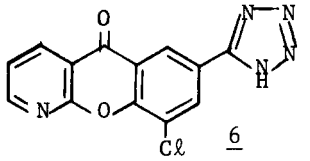
Proxicromil (FPL 57787, 2) provided significant protection at 12mg p.o. against exercise-induced asthma[28] and antigen bronchoprovocation[27] in random double-blind trials in asthmatics. Toxicological findings have resulted in the withdrawal of 2 from further clinical study.[29]

Inhibition of homologous rat PCA was found at 200 mg/kg p.o. with the bis-chromones 3a (-81%) and 3b (-56%). In passively sensitized guinea pigs, 3a (10mg/kg p.o.) significantly inhibited the increase in respiratory rate initiated by i.v. antigen challenge.[30]

The 3-tetrazolyl-chromone AA-344 (4) inhibited PCA in rats (ID_{50}'s 0.28mg/kg i.v., 5.4mg/kg p.o.)[31] and the 3-hour and 8-day homologous PCA in guinea pigs (20mg/kg i.v.). The inhibition by 4 of cAMP-PDE in rat mast cell homogenates was 1000 x more potent than that of theophylline.[32]

In a series of xanthone-3-carboxylic acids (5), only a weak correlation was observed for activities in the rat PCA and the intestinal anaphylaxis (IA) models. Compound 5 (R_1=BuO) was effective in both models at 0.2mg/kg p.o.; however, other analogs (e.g. 5, R_1=H), while equipotent in the PCA were only partially effective in IA. Cyproheptidine, isoproterenol, indomethacin and dexamethasone were also partially effective in IA. A greater involvement of prostaglandins in IA over that in PCA was suggested.[33]

The xanthone Y-12,141 (6) was active (ID_{50} 0.09-0.2mg/kg i.v.; 2.5mg/kg p.o.) in the inhibition of the 48-hour PCA in rats.[34] Active anaphylactic bronchoconstriction in Ascaris-sensitive rats was also inhibited by i.v. or intratracheal administration of 6, with a potency 37 x and 10 x that of DSCG, respectively.



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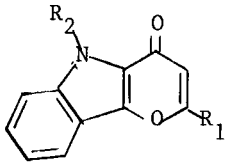
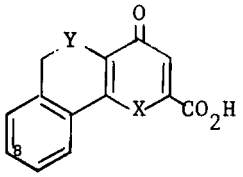
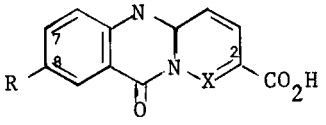


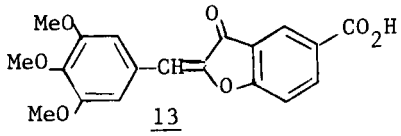
7: R_1=CN_4H, R_2=Et
8: R_1=CO_2H, R_2=Me



9a: X=NH, Y=S
9b: X,Y=O



10 R = OCH_3, X = CH
11 X = N



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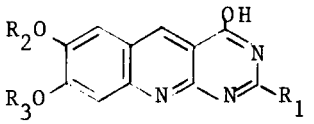


	R_1	R_2	R_3
12a	CO_2Et	OEt	OMe
12b	CO_2Et	OMe	OMe
12c	CH_3	OMe	OMe

The tetrazole (7) was more effective in the rat PCA (100% at 1mg/kg i.p.; 70% at 5mg/kg p.o.) than either the corresponding carboxylic acid or carboxamidotetrazole.[35] Another report[36] defined the PCA activity of 8 as ca. 2 x DSCG and equivalent to that of the indenopyran analog. The most potent analog of the series was the 8-chloro derivative of 9a[37] (ED_{50} 3.3mg/kg i.v.); however oral activity in the rat PCA was only observed with 9b.

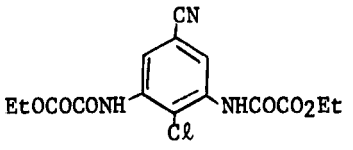
RO21-7634 (10) inhibited antigen-induced rat mast cell degranulation in vitro at 0.06 μM.[38] In vivo, 10 was active in the PCA (ID_{50}'s 1.1mg/kg p.o., 0.2mg/kg i.v.) and in antigen-induced bronchospasm in the rat (ID_{50} 0.2mg/kg p.o.).[39] In the series 11, the 8-chloro derivative provided 91% inhibition at 0.5mg/kg i.v. in the rat PCA.[40] The 8-methyl analog and the 2-chloro-8-carboxylic acid were inactive.

In a large series of 4-oxopyrimido[4,5-b]quinolines,[41] optimum PCA activity (ID_{50}'s 0.002mg/kg i.v.; 0.3mg/kg p.o.) was found in the ester (12a). The 7,8-dimethoxy analog was slightly less potent (ID_{50}'s 0.007 mg/kg i.v.; 1mg/kg p.o.), whereas the corresponding carboxylic acid was inactive at 10mg/kg p.o. The potent oral activity of the 2-methyl representative (12c: ID_{50} 1mg/kg p.o.) was explained in terms of metabolic conversion to the corresponding carboxylic acid.

The most active representative in the rat PCA of a series of benzofuranone carboxylic acids was 13 (ID_{50} 0.96mg/kg i.p.; 1.87mg/kg p.o.). Removal of one or more of the methoxy substituents, or replacement with a larger alkoxy group, reduced activity dramatically.[42]

Lodoxamide ethyl (14; 3mg p.o.) failed to block immediate or late responses in ragweed skin tests on 10 allergic subjects.[43] Gastrointestinal symptoms were reported by a few; however, all subjects complained of flushing and warmth. Contrary to this report is an earlier

study[44] wherein lodoxamide tromethamine inhibited skin reactions in similar challenges. In 12 asthmatics,[45] however, antigen bronchoconstriction was effectively blocked by 14. Other studies and the pharmacological profile of 14 have been reviewed.[46] Another oxamic acid, WY-41,195 (15), has been reported to be active in the rat PCA (ID_{50} 0.3mg/kg p.o.)[47] and in rat passive lung anaphylaxis by oral (ID_{50} 0.1mg/kg) and aerosol (ID_{50}0.3μg) administration.



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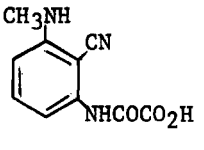

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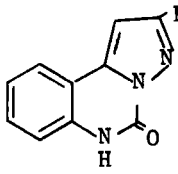


16 R=CO_2H

17 R=CH_2OH

SQ 12903 (16) is > 150 x more potent than SQ 13847 (17) by i.v. administration in the rat PCA. Two thirds of the radioactivity of ^{14}C-17 administered i.v. to monkeys is found as 16 in the plasma after 5 minutes.[48] The inhibition of rat mast cell degranulation by 16 (IC_{50}0.8μM) was compared to that of DSCG (IC_{50} 6μM). Tachyphylaxis and cross-tachyphylaxis with DSCG were also observed.[49]

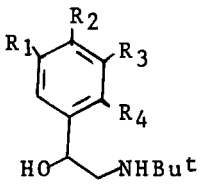
β-Adrenergic Agonists - The concepts and systems utilized to define adenyl cyclase activation by β-adrenergic agonists have been reviewed.[50] The purification of the β-receptor and the characteristics of the antibodies obtained in response to the purified preparations have been described.[51] Autoantibodies to β_2-receptors have been identified in rhinitic and asthmatic patients. It was suggested that their presence may mediate β-adrenergic hyporesponsiveness.[52] A general relationship of hyporesponsiveness with the atopic state has been described.[53]

Clenbuterol (NAB 365, 18; 30 μg tid) was equivalent to terbutaline (19, 5mg tid) in a double-blind, two week study in 24 patients with chronic obstructive lung disease.[54] A low incidence of tremor was found with both drugs.

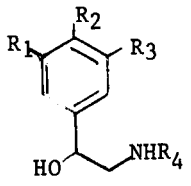
HOKU-81 (21), a metabolite of tulobuterol (20), was 8 x more potent than 20 and twice as potent as salbutamol (22) in relaxing guinea pig tracheal smooth muscle,[55] but equivalent to 22 (i.v. or p.o.) in bronchodilating potency and duration in anesthetized dogs. The ratio of β-selectivity in guinea pig tracheal over atrial tissue was significantly higher for 21 (49.0) than for either 22 (14.5) or 20 (12.6).

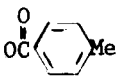
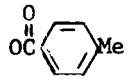
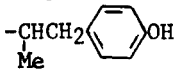
Bitolterol (WIN 32784; 23) was superior in pulmonary function studies in asthmatics to isoproterenol (24) in duration of action.[56] The importance of the prodrug effect was stressed. An increase in heart rate was observed in dogs with 23 (4 & 8mg p.o.), but this effect was significantly less than that found with 19 (4mg) or trimetoquinol (6mg).[57]

The putative selective antiallergic effect of nebulized 25 was assessed in a placebo-controlled study, wherein the effect of the highest non-bronchodilating dose (0.25 to 0.5mg) was measured in allergen-induced bronchospasm. Only marginal protection was observed.[58] An antiallergic effect of topically applied 19 (5mg) and 25 (5mg) was indicated by rhinomanometric measurements following pollen-challenge in 13 asymptomatic patients with hay fever, but was not supported by either rhinoscopy or the patients' opinions.[59]



	R_1	R_2	R_3	R_4
18	Cl	NH_2	Cl	H
19	OH	H	OH	H
20	H	H	H	Cl
21	H	OH	H	Cl
22	CH_2OH	OH	H	H



	R_1	R_2	R_3	R_4
23			H	But^t
24	OH	OH	H	Pr^i
25	OH	H	OH	CMe_2CH_2OH
26	OH	H	OH	
27	OH	H	OH	Pr^i
28	$NHCONH_2$	OH	H	But^t

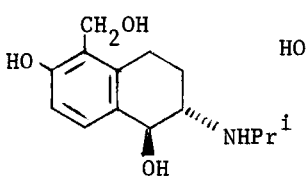
The effect of fenoterol aerosol (26, 0.15mg or 0.3mg) on the upper airways was stronger and of longer duration than on the lower airways in blocking antigen-induced bronchoconstriction in Ascaris-sensitive dogs.[60] The potency and duration of 26 (5mg) and 19 (5mg) were found to be equivalent; however, CNS and musculoskeletal side effects were more prominent with 19.[61] In pulmonary function studies involving 44 asthmatics,[62] 26 (5mg p.o.) was more effective than metaproterenol (27, 10mg p.o.). In a placebo controlled study, intranasal 26 had a marked inhibitory effect on symptoms and changes in nasal airway resistance induced by pollen-challenge in 16 hayfever patients.[63] Another study reported not only a less dramatic effect, but also an increase in nasal airway resistance prior to antigen challenge.[64]

The relative increases induced by 22, 24, 27 and carbuterol (28) in plasma glucose (24 >> 28 > 22 > 27), insulin (24 >> 28 > 27 >> 22) and glucagon (24 >> 28 = 27 = 22) in fasting anesthetized baboons have been described.[65]

AA-497 (29) was 3 x more effective by i.v. and 11 x more effective by intraduodenal administration than 22 in inhibiting histamine-induced increases in pulmonary resistance in anesthetized dogs.[66] In guinea pig tracheal and atrial preparations, 29 was similar to 22 in β_2 selectivity compared to the positive chronotropic action, but 11 x less selective compared to the positive inotropic action.

In a multi-center double-blind crossover study, pirbuterol (30) was judged equivalent to 22, both in efficacy and incidence of tremor.[67] No side effects or difference in bronchodilator effect was observed between 30 (400 µg) and 22 (200 µg) by aerosol administration.[68]

Trimetoquinol (31) partially and reversibly inhibited serotonin-induced contraction of bovine pulmonary vein by a non-β-adrenergic mechanism. cAMP-PDE inhibition was suggested.[69] The analog 32 was 158 x 317 x less potent as a β-agonist than 31 in the guinea pig tracheal and atrial preparations, respectively, but only 13 x less potent



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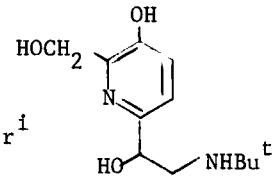

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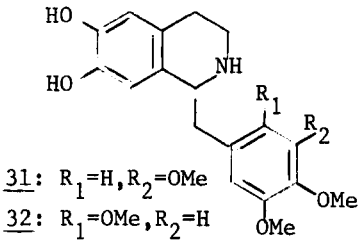
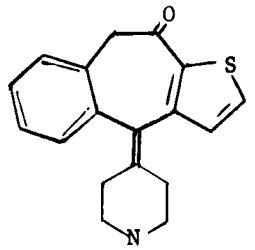


31: R_1=H, R_2=OMe

32: R_1=OMe, R_2=H



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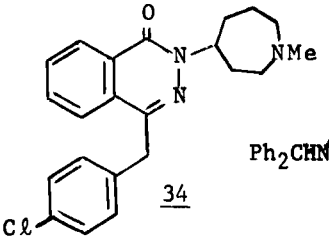

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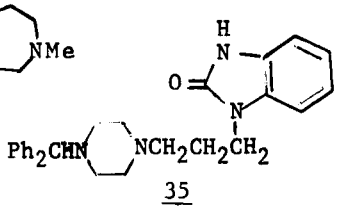

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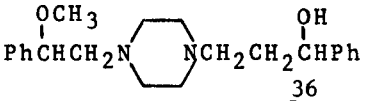

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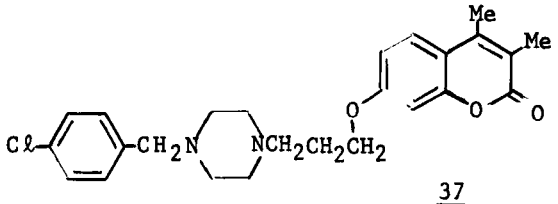


37

in inhibiting arachidonic acid-induced aggregation in human platelet-rich plasma.[70] In contrast to 31, 32 was also effective in a rabbit platelet preparation.

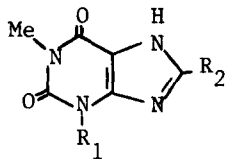
Antiallergic Arylalkylamines - Members of this heterogeneous class were included in an investigation of the effects of antihistamines on anti-IgE induced histamine release from lung fragments in vitro.[71] Release was inhibited (IC_{50}) at low and enhanced (RC_{50}) at high concentrations in a dose dependent manner with all substances tested. Ketotifen (33) and oxatomide (35) had RC_{50}/IC_{50} ratios of 478 and 4738, respectively.

A comprehensive review of the clinical performance of 33 has appeared.[72] In the prophylaxis of bronchial asthma, 33 (1-2mg p.o./day) was judged clinically effective, with transient sedation the only significant side effect.[72, 73, 74] On the negative side, 33 (1 and 2mg b.i.d.) was only slightly effective in atopic asthmatics and ineffective in patients receiving inhaled corticosteroids.[75] Drowsiness limited the study. In the skin of ten atopic individuals, 33 (0.05mg/ml) and clemastine (0.1mg/ml) were effective in inhibiting the wheal to histamine prick testing; however, only 33 significantly inhibited that induced by allergen.[76] DSCG (2mg/ml) was ineffective in blocking wheal formation with both challenges.

Rapid oral absorption of azelastine (34) was observed in rats and guinea pigs with specific distribution to lung tissue.[77] Excretion was primarily via the feces.

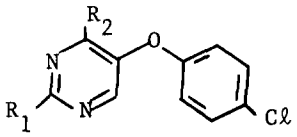
Antigen-induced histamine release from rat peritoneal mast cells and human basophils was blocked by 35 (ED_{50}13µg/ml) but enhanced in unstimulated cells at 32 µg/ml. In a review of the pharmacological behavior of 35, it was concluded[78] that the protective effect of 35 in antigen

challenge is a consequence of the inhibition of mast cell degranulation, rather than blockade of mediator effects. Double-blind studies in allergic rhinoconjunctivitis have found 35 equivalent to,[79] and more effective than,[80] antihistamine therapy. A review[81] of the clinical performance of 35 has concluded that the efficacy of this compound in allergic rhinitis and skin allergies is superior to that observed in asthma.



38 R_1=Me; R_2=H

39 R_1=Ph; R_2=Me



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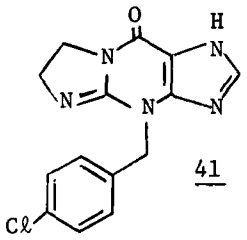
R_1=OH or H

R_2=H or OH



41

Eprozinol (36) inhibited the homologous PCA in rats by i.v. (2-16 mg/kg) or oral (3-50 mg/kg) administration and was equivalent to DSCG in the inhibition of rat mast cell degranulation.[82] Weak inhibiton of reversed cutaneous anaphylaxis in rats and the Arthus reaction in guinea pigs was also reported.

BM 15.100 (37, 20mg p.o.) protected asthmatics from antigen bronchoprovocation in a blind and an open study.[83] Plasma levels of 37 (mean 22.6 µg% after 60 min) were still detectable after 48 hours (mean 1.4 µg%). No side effects were observed.

Phosphodiesterase (PDE) Inhibitors - Histamine-induced bronchoconstriction in guinea pig lung lobes was coincident with increases in cAMP and cGMP levels. Increases of the same magnitude were effected by perfusion of the lobes with theophylline (38, 1mg/ml) or isobutylmethylxanthine (100µg/ml); however, in this instance no further increase or bronchoconstriction was observed on histamine challenge.[84] The blockade by indomethacin of the protective effect of 38 or dibutyryl cAMP in anaphylactic shock in rats suggests increases in PGE levels subsequent to rises in cAMP.[85]

D-4026 (39) was 10 x more potent than 38 in inhibiting IgE-mediated histamine release from human leukocytes[86] and synergistic with histamine H_2-receptor agonists. The aryloxypyrimidinones (40;60mg/kg p.o.) inhibited histamine-induced bronchoconstriction in guinea pigs.[87] A shift of the substituent to the m-position of the aryl ring or a replacement of the aryl ether by a thioether linkage reduced activity significantly.

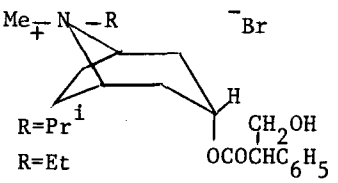
The dihydroimidazopurinone (41) was slightly more active (ID_{50} 34mg/kg p.o.) than 38 (ID_{50} 42mg/kg p.o.) in the rat PCA but significantly more effective in blocking methacholine (ID_{50} 5.2mg/kg intraduodenally) or antigen-induced (ID_{50} 0.9mg/kg intraduodenally) bronchospasm in rats.[88]

Quantum chemical calculations on flavanoids suggest that the pyrimidine ring of cAMP is mimicked by the pyranone ring.[89] The relative potencies of a large group of pyranones in inhibiting 15-hydroxy-prostaglandin dehydrogenese (15-OH-PG-DH), cAMP-PDE and cGMP-PDE derived from guinea pig lung have been reported.[90] Representative examples are as follows (IC_{50}'s 15-OH-PG-DH; cAMP-PDE; cGMP-PDE µM): DSCG(350; 650; 90), quercetin (15; 18; 8.4) and baicalein (3; 9.7; 40).

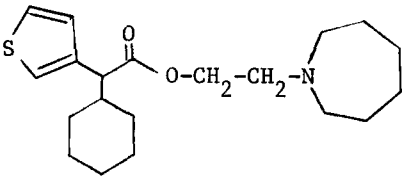
Corticosteroids - Corticosteroids have been shown[91,92] to stimulate the synthesis and cellular release of a proteinaceous inhibitor(s) of phospholipase A_2. The two inhibitors which have been defined differ dramatically in their molecular weights, i.e., 40,000[91] and 15,000,[92] although their origin and cellular effects are similar.

The utility of flunisolide in rhinitis has been reviewed.[93] Intranasal flunisolide (200 µg/day)[94] and beclomethasone dipropionate (400µg/day)[95] were judged clinically effective and free of significant side effects in this indication. Biopsy of the nasal mucosa following a 3-month treatment with flunisolide[96] did not reveal any histological abnormalities. Intranasal DSCG (10mg) was less effective than beclomethasone dipropionate (100µg) in allergic rhinitis.[97] The relaxation and potentiation of isoproterenol effects in smooth muscle by glucocorticoid succinates is primarily due to the succinate and not the glucocorticoid moiety.[98]

Anticholinergics - A review of the therapeutic utility of ipratropium bromide (42) has appeared.[99] Synergism of 42 with DSCG in exercise-induced asthma[3] and with salbutamol in asthmatic and bronchitic patients[100] has been described. Oxitropium bromide (Ba 253 (43), 0.2mg inhaled) provided bronchodilation of long duration (10 hrs) in a controlled study with 15 patients having stable obstructive lung disease.[101]



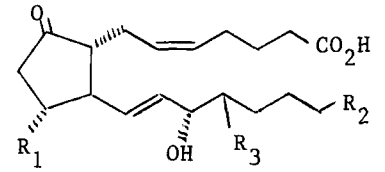
42 R=Pr^i
43 R=Et



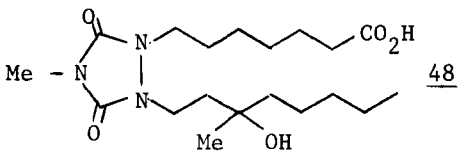
44

The effective blockade of methacholine-induced bronchospasm in 19 asthmatics by cetiedil (44) was attributed to its anticholinergic and smooth muscle relaxant properties.[102]

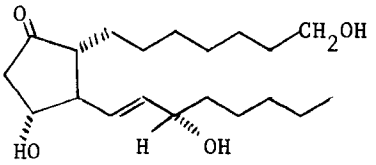
Prostaglandins - A review of the structure-activity relationships of prostaglandins on human respiratory smooth muscle has appeared.[103] The bronchodilating effect of 45 on histamine-induced bronchoconstriction in guinea pigs was 0.15 x that of PGE_2 but of longer duration and free of cardiovascular effects.[104] Saturation at the Δ^{13} double bond did not alter potency.



45: R_1=$HOCH_2CH_2S$-; R_2=CH_3; R_3=H
46: R_1=OH; R_2=$CH{=}C(CH_3)_2$; R_3=H
47: R_1=OH; R_2=CH_2OCH_3; R_3=CH_3



48



49

CS-412 (46) was equivalent to PGE_2 by aerosol and 2 x more potent i.v. against serotonin-induced bronchoconstriction in anesthetized cats.[105] Bronchoconstriction by 46 in cat bronchi having normal tone was 30 x less than PGE_2. Increases in specific airway conductance in 5 asthmatics upon

inhalation of 46 (50-200 μg) were dose-related. Irritable sensations and hypotension were absent. YPG-209 (47) was 150 x more potent than DSCG in the rat PCA and inhibited the 8-day PCA in guinea pigs (ED_{50} 3.6 μg/kg i.v.).[106] Histamine and serotonin hypersensitivity in B. pertussis-treated mice was also blocked (10 μg/kg p.o.). The α-side chain and the methyl substituents in 48 are prerequisites for significant inhibition of serotonin-induced bronchoconstriction in guinea pigs (IC_{50} 3.9 μg/kg i.v.).[107] In in vitro and in vivo models, TR-4161 (49) was 100-1000 x less irritating than PGE_1 but equivalent in bronchodilator potency .[108]

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Chapter 8. Antihypertensive Agents

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General - A fuller report of the Australian therapeutic trial in mild hypertension confirms that drug treatment can reduce morbidity and mortality from cardiovascular disease. The most significant benefit was in preventing cerebrovascular events but, some protection from ischemic heart disease was also apparent.[1] A smaller Norwegian trial in mildly hypertensive men has also shown that treatment affords protection from pressure-related complications, such as stroke and congestive heart failure.[2] However, no benefit emerged with respect to coronary heart disease, and speculation continues that the adverse effect of thiazide diuretics and β-blockers on lipid metabolism could counteract any reduction in coronary risk conferred by blood pressure control.[2-4] Nevertheless, the results of these studies and the Hypertension Detection and Follow-up Program should encourage the treatment of milder forms of the disease,[5,6] but it is also apparent that further advances in therapy are required. Multiple-drug therapy is often necessary, side effects are by no means infrequent, and, as shown by the Australian trial, long-term compliance is important.[7]

Etiology - The relative contributions of environmental and genetic factors to hypertensive disease may emerge from studies of different racial groups.[8] The prevalence of hypertension is greater in black than in white Americans and is more commonly of the low renin type. This has led to the proposal that the former population has evolved an enhanced capacity to retain salt and water which, though appropriate to a sub-tropical habitat, proves disadvantageous on exposure to a high dietary salt intake.[8-10] Salt retention and hence fluid expansion is commonly thought to raise blood pressure through a process of autoregulatory adaptation. However, the blood pressure rise could be due to a natriuretic hormone (NH) released primarily to promote salt excretion.[10] Previously, it had been proposed that such a hormone with Na^+, K^+-pump inhibitory activity would raise vascular tone.[11] As a further development, it is argued that an inherited tendency to retain salt, corrected by NH, is an important cause of essential hypertension (EH), so that the renal inadequacy is obscured, but blood pressure is left elevated.[12] In fact, there is now good evidence that renal salt excretion is partly regulated by one or more NHs with cardiac glycoside-like properties. Small peptides, which displace digoxin from specific digoxin antibodies, have been separated from dog plasma. These peptides are thought to be derived from an inactive circulating precursor, and their concentration is much increased by volume expansion.[13] Additionally, it is reported that the concentration of a human plasma constituent, which inhibits renal Na^+-K^+-ATPase, is increased by raising salt intake.[14] Also of interest is that atrial extracts produce a profound saliuresis in the rat, while extracts from ventricular tissue are inactive. Thus, atria, which are known to be monitoring sites for intravascular volume, may release a factor which regulates renal function.[15]

Studies with erythrocytes from hypertensive patients and their offspring have led to the proposal that EH may be due to a reduced capacity

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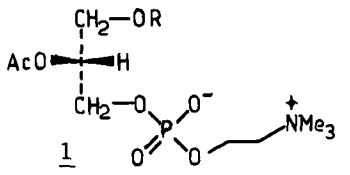
to extrude intracellular Na^+ through an inherited disorder of the furosemide-sensitive Na^+, K^+-co-transport system.[16] Also bearing on this is the report of an increase in the maximum rate of erythrocyte Na^+, Li^+-counter-transport in hypertensive patients and their first-degree relatives,[17] since this transport system probably operates through the same pathway as Na^+, K^+-co-transport.[18] An alternative explanation however, is that these in vitro abnormalities may reflect prolonged Na^+, K^+-pump inhibition in vivo.[19] In support of this conclusion, the ouabain-sensitive component of the Na^+ efflux rate constant of leucocytes from hypertensive patients is significantly lower than that of normotensive subjects, and when leucocytes from these latter individuals are preincubated with serum from hypertensive patients, the efflux rate constant is reduced to levels found in hypertensives. Consequently, the inherited abnormality in Na^+ transport in EH may be mediated by a circulating transport inhibitor and not due to an inherent cellular defect.[20]

It is proposed that the kidney secretes an antihypertensive hormone which is believed to be a neutral glyceryl ether. Hydride reduction and acetylation of renomedullary extracts has led to the isolation of 1.[21,22] This material produces prolonged antihypertensive responses at low oral doses in rats[21] and rabbits without alteration in platelet and white cell counts,[22] despite its identity to Platelet Activating Factor.[23]



1

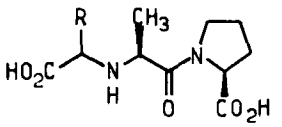
R = C16:0, 67%; C16:1, 16%
C18:1, 11%; C18:0, 4%

Renin-Angiotensin, Kallikrein-kinin System - With the advent of angiotensin-II (A-II) antagonists and angiotensin converting enzyme (ACE) inhibitors, the potential of direct renin blockade is of considerable interest. Dog renin-specific antibody and Fab fragment have been obtained and shown to reduce blood pressure in normotensive salt-depleted and renally hypertensive dogs.[24] However, potentially more useful as an investigative tool in man is the decapeptide renin substrate analog, Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys, which inhibits human renin and lowers blood pressure in normotensive salt-depleted and renally hypertensive monkeys.[25] The preparation and renin inhibitory activity of pepstatinyl-Arg-O-methyl ester has also been further described.[26]

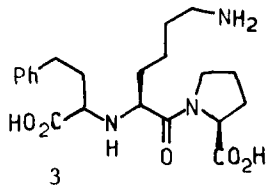
The Cardiovascular-Renal Advisory Committee of the FDA has recommended the approval of captopril (Capoten®) for the treatment of refractory hypertension. Clinical mode of action studies with captopril continue to give rise to conflicting conclusions. Some hold that activity is due mainly to suppression of the renin-angiotensin-aldosterone system.[27] However, there is now growing evidence that augmented plasma kinin levels can contribute,[28] especially after chronic treatment.[29] Of note is that in a single-dose comparative study in patients plasma kinin levels rose after teprotide but not after captopril, suggesting a mechanistic distinction between these compounds.[30] Prostaglandins do appear to make a contribution to the acute response to captopril, particularly when the renin-angiotensin system is suppressed.[31] Recent studies have shown that renin lowers blood pressure in captopril treated dogs by releasing bradykinin and PGI_2-like substances. Thus, the rise in plasma renin activity after captopril may contribute to its antihypertensive action.[32]

The screening,[33] development, SAR and mechanism of action of ACE inhibitors have been reviewed.[34] It has been commented that the range of side effects associated with captopril is similar to that of d-penicillamine, which suggests that the thiol function may be responsible.[35] A highly interesting and thiol-free series of N-carboxymethyl dipeptide

analogs, such as 2a-c and 3, with in vitro ACE inhibitory activity in the nanomolar range has been reported. Compound 4a (MK-421), 4b and the more active diastereoisomer of 3 were all potent and long-acting ACE inhibitors orally in the dog, and MK-421, which is hydrolyzed to 4b in vivo, has been selected for clinical evaluation.[36] High ACE inhibitory activity has been described for N-phosphoryl-Ala-Pro (5)[37] synthesized by analogy with phosphoramidon, a potent inhibitor of the zinc endopeptidase thermolysin. Despite hydrolytic susceptibility, 5 lowered blood pressure when infused in hypertensive rats.[38] Compound 6 was found to have greater ACE inhibitory activity than the corresponding tripeptide Bz-Phe-Gly-Pro and captopril, but potency was much reduced in the guinea-pig ileum assay, possibly due to poor cell penetration.[39]



2a, R=$(CH_2)_2CH(CH_3)_2$
b, R=$(CH_2)_2Ph$
c, R=$(CH_2)_4NH_2$



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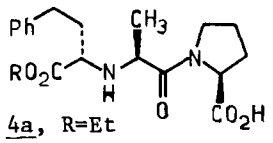
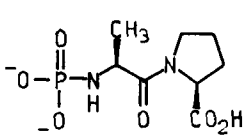


4a, R=Et
b, R=H



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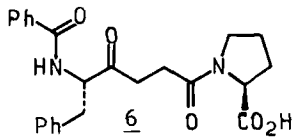

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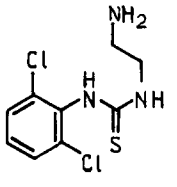
Orally administered hog pancreatic kallikrein has lowered blood pressure in hypertensive patients with low urinary kallikrein levels. The response was accompanied by an increase in glomerular filtration rate and urinary excretion of endogenously formed kallikrein.[40] However, there is disagreement that decreased urinary kallikrein levels are a general feature of EH, but are due to a degree of renal insufficiency.[41]

Centrally Acting Drugs - Mechanistic and therapeutic aspects of clonidine have been reviewed.[42] Propranolol is reported to produce virtually no antihypertensive response in clonidine treated patients. This lack of effect may be caused by an increase in peripheral resistance due to β_2-adrenoceptor blockade, which offsets the hemodynamic response to a reduced cardiac output, as a significant reduction in blood pressure was obtained on substituting propranolol by the β_1-selective blocker, atenolol.[43] However, studies in SHR, where propranolol has been shown to antagonize the antihypertensive action of clonidine, suggest a central site of interaction.[44] Clinical studies with tiamenidine,[45] lofexidine,[46] guanabenz[47] and guanfacine[48] reveal similar antihypertensive and side effect profiles to clonidine. Sedation is claimed, however, to be less prominent with lofexidine[46] and guanfacine,[49] and, possibly due to a gradual offset of action, the latter compound may also have an advantage with respect to the rebound withdrawal syndrome.[50]

The thiourea 7 is orally active in rats and dogs and produces less sedation in the dog and monkey than clonidine.[51] Azepexole (B-HT-933, 8) exhibits a similar pharmacological profile to clonidine and, although some 50x less potent,[52] has significantly greater selectivity for α_2- as opposed to α_1-adrenoceptors.[53] A more potent analog B-HT-920 (9) has recently been described.[54]



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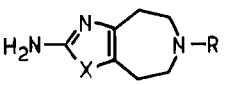


8 X=O, R=Et
9 X=S, R=Allyl

Stimulation of inhibitory presynaptic opiate receptors suppresses transmitter release from peripheral sympathetic neurones,[55] which may in fact co-release opioid peptides.[56] Intracerebroventricularly administered Leu-enkephalin produces a greater rise in blood pressure in SHR than in normotensive animals.[57] Naloxone does not antagonize the hypotensive action of clonidine in normotensive man[58] and normotensive rats, but an inhibitory effect is evident in SHR.[59] Thus, a central opioid pathway may be involved in raising sympathetic tone in hypertension and partly mediate the antihypertensive action of α_2-adrenoceptor agonists.

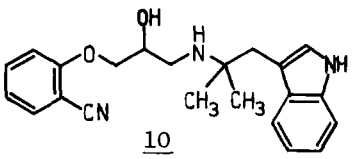
The involvement of histamine H_2-receptors in mediating the antihypertensive action of clonidine is still unclear. Though clonidine has low affinity for ^{3}H-cimetidine specific binding sites, it now appears that the latter ligand may not directly label histamine H_2-receptors.[60]

Adrenergic Blocking Agents - Most β-blockers have similar antihypertensive efficacy, but cardioselective hydrophilic agents are less likely to affect carbohydrate metabolism, renal function or to cause CNS disturbances.[61,62] Thus, β_2-receptors control to some extent glycogenolysis and renal blood flow, and blockade may compromise both functions.[63] The relative ineffectiveness of alprenolol[64] and atenolol[65] in black patients may reflect the prevalence of low-renin hypertension in this patient population.[8]

In man, propranolol lowers blood pressure and increases the excretion of the major urinary metabolite of $PGF_{2\alpha}$, and both effects are antagonized by indomethacin.[66] Although this suggests prostaglandin involvement in the antihypertensive activity of propranolol, in other studies, neither atenolol[67] nor propranolol[68] affected the urinary excretion of $PGF_{2\alpha}$ or PGE_2. In addition, besides inhibition of cyclooxygenase, indomethacin has other activities, and effects on sodium balance, β-receptor number and function should be considered.[69] The antihypertensive effect of propranolol in captopril-treated patients is not accompanied by a further decrease in plasma AII levels. Thus, attenuation of the renin-angiotensin system is not involved, but rather sympathetic activity appears to be the major factor in determining response to the β-blocker.[70] This may also be the case more generally, and the usefulness of determining renin status may be that it provides a marker of sympathetic tone.[71] The presynaptic action of β-blockers in antagonizing both the AII potentiation of sympathetic nerve activity[72] and adrenaline-mediated transmitter release[73] may not involve β-receptors.[74] Chronic propranolol treatment elevates β-receptor density on human lymphocytes,[75] and there is a marked increase in isoproterenol-induced cyclic-AMP production after four weeks.[76] These observations support an earlier hypothesis that β-blockade allows vascular β-receptors to recover their original sensitivity and vasodilator response to natural transmitters.

Prevention of the development of hypertension in SHR by propranolol could be due to central inhibition of sympathetic vasomotor tone,[77] and furthermore, the drug does accumulate in specific areas of rat brain involved with cardiovascular control.[78] However, a central site of action is inconsistent with the fact that the dose of propranolol required to lower blood pressure in SHR is identical for s.c. and i.c.v. administration, although one hundred-fold higher brain levels were achieved in the latter case.[79] The marked elevation in plasma dopamine levels after chronic metoprolol treatment in man may contribute to the increased cardiac output and decreased peripheral resistance usually seen after long term β-blocker administration.[80]

Efforts to improve on currently available β-blockers continue to focus on the introduction of a component of vasodilator activity. Thus, various heterocyclic analogs of MK761 have dual activities, but none is superior to the parent compound.[81] Bucindolol (MJ 13,105, 10), is a member of a series of indolyl-t-butyl substituted phenoxypropanolamines with combined β-blocking, vasodilator and α-antagonist activities, which lowers blood pressure in rats and dogs.[82,83] The tachycardia seen with bucindolol in the dog is not observed in man and is only partly due to β-agonist activity.[84] Prizidilol (SK&F 92,657) possesses both direct vasodilator and β-blocking properties and lowers blood pressure in patients with little effect on heart rate during chronic treatment.[85]



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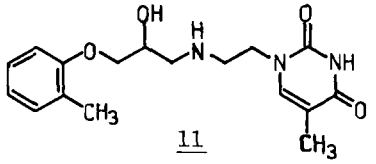

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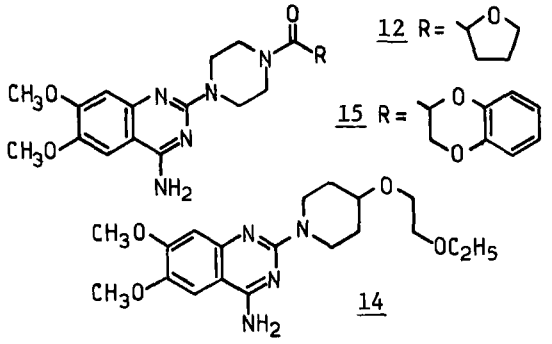
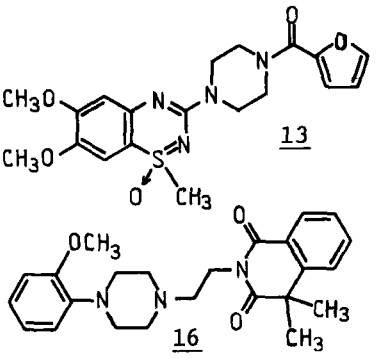
In contrast to propranolol, labetalol does not elevate plasma triglycerides[86] or reduce renal blood flow.[87] This may be a consequence of α-blocking properties, although for the renal effect, partial β_2-agonist activity may also be relevant.[88] The α- and β-antagonist properties of both labetalol and medroxalol (RMI 81,968A) reside in separate stereoisomers, and dual activity is not sufficiently balanced in the individual molecular entities.[89,90] Both compounds also possess direct vasodilator activity,[91] and in man, combination of medroxalol with hydrochlorthiazide provides effective antihypertensive therapy.[92] The α/β-blocker primidolol (UK-11,443, 11) produces a clinically significant reduction in blood pressure with negligible side effects.[93]

The increased peripheral resistance which characterizes most hypertensives may be the result of unopposed α-mediated vasoconstriction as β-receptor sensitivity declines with age or after prolonged sympathetic overactivity.[94] Thus, selective blockade of postsynaptic α_1-receptors by prazosin lowers blood pressure, with beneficial hemodynamic results,[95] and homeostatic reflexes are largely preserved, since postsynaptic α_2-sites are still able to respond to sympathetic activation.[96] However, the significance of the postsynaptic selectivity of prazosin[97] and the physiological role of presynaptic α_2-receptors have been questioned.[98] The lack of tachycardia after prazosin administration may not be due to impaired sympathetic responses[99] but may simply be the consequence of decreased venous return.[100] In anesthetized cats, prazosin inhibits central sympathetic discharge, without altering heart rate.[101] On the other hand, unchanged CSF levels of catecholamine metabolites do not support a central mechanism for prazosin in man.[102] Chronic prazosin administration reduces total serum cholesterol, LDL and VLDL cholesterol and total triglycerides,[103] while in diabetic hypertensives, glucose tolerance is improved.[104] Prazosin is effective in patients refractory to hydralazine.[105]

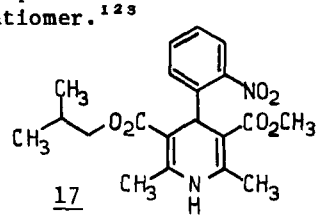
The antihypertensive activity of trimazosin is accompanied by an increase in renal blood flow, and this may be particularly useful when kidney function is impaired.[106] Initial reports with tiodazosin indicate that the drug lowers blood pressure in man and has a plasma half-life of 8 hr.[107] Terazosin (Abbott 45,975, 12) and the benzothiadiazine 1-oxide derivative (13) are similar to prazosin in lowering blood pressure in SHR.[108,109] In addition, 13 has been shown to be a potent postsynaptic α-antagonist. In anesthetized animals, UK-18,596 (14), UK-33,274 (15) and AR-C239 (16) lower blood pressure by blockade of vascular postsynaptic

α_1-receptors, although in the latter case, central sites and metabolites could also be involved.[110,111]





Vasodilators - Nifedipine lowers blood pressure in hypertensive rats[112] and man[113] more effectively than in normal controls, and this may reflect differences in compensatory baroreceptor sensitivity, as well as correction of abnormalities in calcium transport.[114] It has been suggested that the antihypertensive activity of calcium antagonists results from selective reduction of the vascular tone controlled by postsynaptic α_2-receptors.[115] Thus, vasoconstriction mediated via these receptors is said to require an influx of extracellular calcium, although alternative mechanisms have been proposed.[116] The clinical efficacy and tolerance of nifedipine can be enhanced by addition of α-methyldopa,[117] clonidine[118] or a β-blocker.[119] Nisoldipine (Bay k 5552, 17)[120,121] and H154/82 (18) are more potent vasodilators than nifedipine and lower blood pressure in animals and man.[122] Resolution of nicardipine (YC-93) indicates that the (+) enantiomer is the more potent vasodilator and has a longer duration of action than the (-) enantiomer.[123]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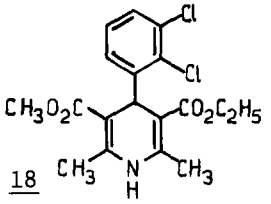
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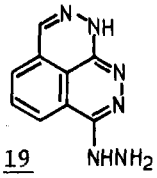
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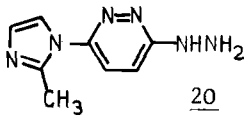
The lack of reflex increases in heart rate, renin release or fluid retention following reduction of blood pressure with verapamil in hypertensive patients may be due to attenuation of calcium-dependent transmitter release from sympathetic nerve endings.[124] The diuretic agent indapamide has also been classified as a calcium antagonist,[125] although there appears to be little direct evidence. However, in DOCA dogs, the antihypertensive activity of the compound does appear to be mediated via a mechanism independent of diuretic-induced fluid reduction.[126]

Chronic minoxidil therapy is particularly effective in refractory patients,[127] and severely compromised renal function can be improved.[128] Clinical evaluation of GPA 1595 (19) indicates a profile similar to hydralazine,[129] while the imidazolylpyridazine derivative (20) is more potent than dihydralazine in SHR.[130] The antihypertensive effects of cadralazine (ISF 2469) may be mediated via a hydrazino metabolite.[131] The vasodilator P 1134 (21) shows long-lasting antihypertensive activity in animals and man,[132] and the 1,2,4-triazolo[1,5a]pyrimidine derivative (22) also lowers blood pressure in SHR.[133]



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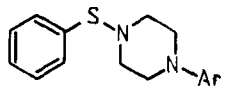
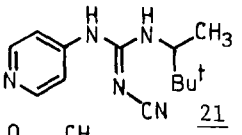


23, Ar=Phenyl, 2-pyridyl



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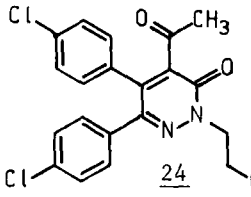

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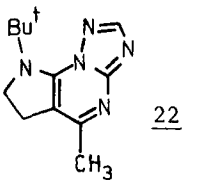

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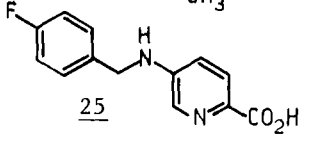

25

Miscellaneous - The long-acting antihypertensive activity of molsidomine in man may be due to dilatation of the capacitance vessels and reduced cardiac output.[134] MK447 lowers blood pressure in SHR, in contrast to closely related saliuretic analogs, and antagonism by indomethacin implicates prostaglandin involvement in its mechanism of action.[135] The sulphenamides (23) and pyridazinone derivative (24) are orally active in SHR although limited bioavailability was encountered in the latter case.[136,137] The synthesis of kukoamine A, the active component of the oriental antihypertensive preparation "jikoppi," has been reported.[138] In contrast to ALDOMET®, α-monofluoromethyldopa is not metabolized to the corresponding catecholamines but instead inhibits noradrenaline synthesis at the decarboxylase step, thereby depressing sympathetic function.[139] Both this compound and the fusaric acid analog (25) lower blood pressure in rats and dogs but appear to be poorly tolerated on chronic administration.[140,141] 3-Deaza-adenosine[142] and the marine natural product 1-methylisoguanosine[143] (doridosine)[144] lower blood pressure in rats, possibly by activation of either adenosine-sensitive adenyl cyclase[143] or presynaptic purinergic receptors.[145]

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Chapter 9. Agents for the Treatment of Peptic Ulcer Disease

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General - The introduction of efficacious drugs for the treatment of peptic ulcer disease has renewed the interest both in the discovery of new therapeutic agents and in the research activity associated with understanding the etiology of this disease. The newer therapeutic agents useful in the treatment of ulcer disease have been the subject of several review articles.[1-3] A text,[4] and several symposia[5,6] on the etiology and treatment of peptic ulceration have been published recently. A collection of papers relating to clinical studies of antiulcer drugs has also been issued.[7]

The major direction being pursued in developing new antiulcer drugs involves effective inhibition of gastric acid secretion as exemplified by the H_2-receptor antagonists. Reviews have been published concerning mechanisms involved in controlling gastric secretion.[8,9] Another approach which is receiving considerable attention involves the discovery of agents which protect the gastrointestinal tract from damage induced by noxious agents.[10] Of special interest in this regard are the prostaglandins and their analogs.[10] The relevance of cytoprotection to the ulcer healing process remains to be determined.

H_2-Receptor Antagonists - The unprecedented success of cimetidine (1)[11] as a therapeutic agent has provided the impetus for the development of second generation H_2-receptor antagonists having greater potency and specificity. The function of histamine H_2-receptors in modulating gastric acid secretion was reviewed.[12,13] An historical account of the development of cimetidine was published,[14] and its use in the management of peptic ulcer disease was reviewed.[15]

A furan derivative, ranitidine (AH 19065, 2), is 10 times as potent as cimetidine *in vitro*.[16] The action of ranitidine *in vitro* is selective; high concentrations do not affect β-adrenoceptor, H_1-receptor, or muscarinic-receptor mediated responses.[17] In the dog, the gastric antisecretory effects of ranitidine, cimetidine, and metiamide (3) were compared: when gastric acid was stimulated by histamine, ranitidine was 4.3 times as potent as cimetidine and metiamide when given orally, whereas for pentagastrin stimulation, ranitidine was 5 and 10 times more potent than cimetidine and metiamide, respectively. The effects of ranitidine on stimulated gastric acid secretion in normal volunteers were evaluated in several studies: ranitidine was effective in reducing acid secretion stimulated by histamine and pentagastrin, and was 6-8 times as potent as cimetidine.[18-21] In patients with duodenal ulcers, ranitidine effectively healed the ulcers when administered in 100 mg doses twice daily, and generally was five times as potent as cimetidine.[22-24] Metabolically, ranitidine undergoes N-oxidation and N-demethylation of the 3° amino group and sulfoxide formation.[25] No effects on prolactin secretion have been observed, demonstrating that ranitidine is neither a dopamine antagonist nor agonist at the pituitary level.[26]

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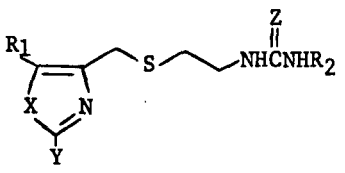
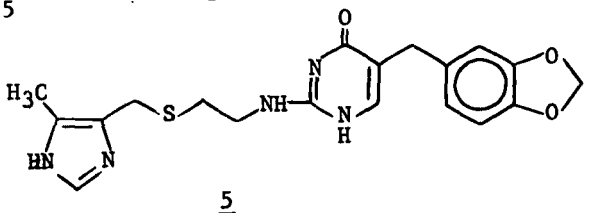
Tiotidine (ICI 125211, 4), a guanidino thiazole, was the most potent H_2-receptor antagonist reported through 1980, being at least 10 times as potent as cimetidine both *in vitro* and *in vivo*.[27] Tiotidine has been proposed as a useful tool for studying the effects of histamine on cardiac function and dysfunction.[28] In duodenal ulcer patients, tiotidine reduced nocturnal gastric acid secretion at doses as low as 25 mg.[29] A review compared the pharmacology of cimetidine, ranitidine, and tiotidine.[30] The occurrence of mucosal lesions, which apparently are intramucosal carcinomas, in rats administered doses of tiotidine which were greater than 150 mg/kg/day for one year led to the suspension of clinical trials with this agent.[31] The tumorigenicity may or may not have been an effect of high intragastric pH over a long period of time.[32]

Oxmetidine (SKF 92994, 5), which contains a (3,4-methylenedioxybenzyl)pyrimidone group replacing the cyanoguanidine of cimetidine, was more potent than cimetidine *in vitro* and equipotent *in vivo*.[33]

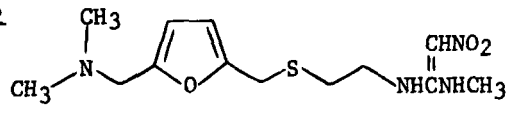
Etintidine (BL-5641, 6), wherein the N-methyl of cimetidine is replaced by a propargyl group, is intermediate in potency between cimetidine and ranitidine. It also exhibited weak anticholinergic activity.[34] Clinically, etintidine was slightly more effective than cimetidine as an inhibitor of meal-stimulated gastric acid secretion in patients with duodenal ulcers.[35]





5

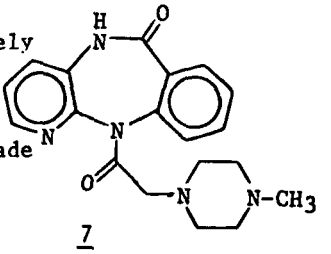
	X	Y	Z	R_1	R_2
1:	NH	H	NCN	CH_3	CH_3
3:	NH	H	S	CH_3	CH_3
4:	S	$N{=}C(NH_2)_2$	NCN	H	CH_3
6:	NH	H	NCN	CH_3	$CH_2C{\equiv}CH$



2

Anticholinergic Agents - Fentonium bromide and cimetidine were compared for efficacy in healing duodenal ulcers alone and in combination. The combination was slightly better than either drug alone.[36]

Pirenzepine (LS 519, 7) is a new tricyclic antiulcer agent with a log P of 0.1 which effectively prevents it from crossing the blood-brain barrier.[37-39] It is a selective antimuscarinic agent which distinguishes between different subclasses of muscarinic receptors, favoring blockade of the gastric secretagogue action of muscarinic agonists compared with cardiac effects.[40,41] In a double-blind controlled study, pirenzepine was as effective as cimetidine in healing duodenal ulcers.[42] A collection of papers[43] and a symposium[44] concerning pharmacokinetic and clinical studies with pirenzepine have been published.

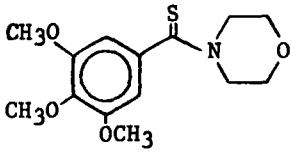


7

Other Antisecretory Agents - Apart from the H_2-receptor antagonists, there are a number of compounds undergoing antiulcer evaluation which reduce gastric acid secretion by mechanisms other than competitive H_2-receptor antagonism.

Trithiozine (ISF 2001, 8) is a member of a series of thioamides exhibiting gastric antisecretory activity in the Shay rat and antiulcer activity in the mouse.[45] Clinically, trithiozine is well tolerated[46] and is as effective as cimetidine in treating patients with duodenal ulcer.[47,48] Bioavailability studies demonstrate a measurable plasma level in man exceeding 5 hours.[49]

Another thioamide, tiquinamide (Wy 24081, 9), is a member of a series of tetrahydroquinoline thioamides with gastric antisecretory activity.[50,51] It inhibits basal as well as carbacol, betazole, and pentagastrin-stimulated gastric acid secretion in rats and dogs.[52] Tiquinamide is a weak H_2-receptor antagonist on guinea pig atrial tissue and is devoid of anticholinergic activity. It may affect some fundamental ion transport process beyond the H_2-receptor on the parietal cell.[52]



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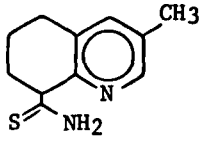
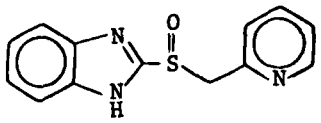


9

RMI 14451A, a structural analog of clonidine, is slightly more potent than cimetidine in the Shay rat and was ineffective in the perfused rat stomach at 20 mg/kg ip against gastric acid secretion stimulated by histamine, pentagastrin, bethanecol, and dibutyryl cyclic-AMP. It did inhibit 2-deoxy-D-glucose-induced gastric acid secretion and may act by interferring with vagal transmission to the stomach.[53]

Timoprazole (H 83/69, 10)[54] is representative of a class of benzimidazoles which are potent noncompetitive inhibitors of histamine-stimulated gastric acid secretion.[55,56] Studies on the isolated guinea pig gastric mucosa show that timoprazole inhibits dibutyryl cyclic AMP-stimulated acid secretion, suggesting that the inhibition occurs within the parietal cell at a site peripheral to the cyclic nucleotide step.[57]

Pifarnine (U-27, 11)[58] has been shown to prevent experimental ulcers in animals induced by pylorus ligation, stress, reserpine, or histamine.[59] In a double-blind trial of patients with duodenal ulcers, 64% were healed with a 150 mg/day dose of pifarnine vs. 19% for placebo.[60]



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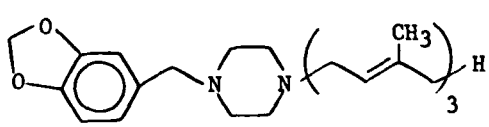
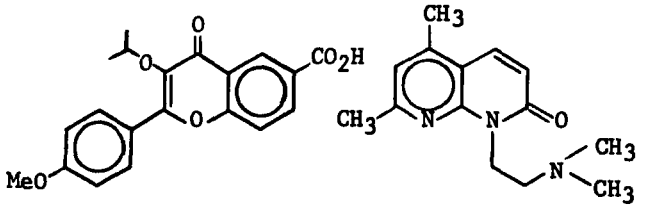


11

The flavone 12 is a member of a class of compounds studied for their antiallergy activity, which inhibits histamine-stimulated gastric acid secretion in the rat. Orally, 12 was half as potent as metiamide. These compounds have neither H_2-receptor antagonistic nor anticholinergic properties.[61] There is one report that disodium cromoglycate inhibited reserpine-induced ulcers in rats.[62]

A series of 1,8-naphthyridin-2(1H)-ones typified by 13 is reported to reduce pentagastrin-induced gastric acid secretion in dogs, but not histamine-induced secretion. Many of the compounds in this class were H_2-receptor antagonists on the guinea pig atrium and rat uterus.[63]

The cyclopentapyrroles 14-16 showed pronounced antisecretory activity in the pylorus-ligated rat and on histamine-stimulated gastric acid secretion in dogs. Both 14 and 15 caused bone marrow depression in monkeys and 16 induced convulsions in dogs.[64]



12

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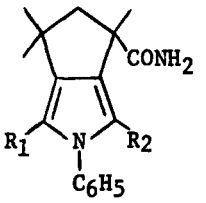


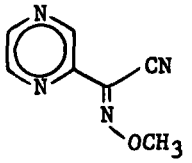
14: $R_1=R_2=CH_3$

15: $R_1=CH_3$; $R_2=CH_2OH$

16: $R_1=CH_2OH$; $R_2=H$

CF 19415 (17)[65] is representative of a series of α-alkoxyimino-nitriles which are antisecretory and increase the gastric mucus barrier.

A series of benzylureas and thioureas inhibited gastric ulceration induced by indomethacin, stress, and pylorus ligation.[66] ITA 312 (18) is a clinical candidate from this class of compounds.



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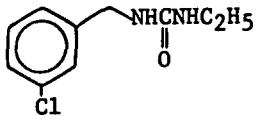


18

Prostaglandins - The mechanism by which prostaglandins (PGs) exert their antiulcer effects may involve both inhibition of gastric acid secretion and/or cytoprotection. The general properties of PGs have been reviewed.[67,68] All PGs are cytoprotective to varying degrees, but there is no direct correlation between cytoprotective and antisecretory effects.[10,69,70] The cytoprotective mechanism has not been defined, but may include stimulation of mucus secretion,[70] stimulation of sodium transport,[71] activation of adenyl cyclase,[70] stimulation of mucosal blood flow,[70] or stimulation of chloride transport.[72] The physiological role of PGs in peptic ulcer disease also remains uncertain.[73] Plasma PG concentrations in patients with duodenal ulcer failed to correlate with parameters of gastric secretion and were unaffected by H_2-receptor blockade or the ulcer activity.[74] Isolated parietal cells were found to produce PGs suggesting that endogenous PGs may play a physiological role in regulating gastric acid secretion.[75]

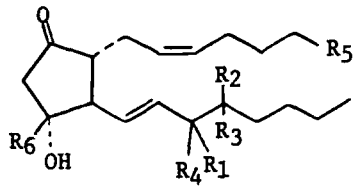
(15R)-15-Methyl PGE_2 (arbaprostil, 19), in 50-100 μg dose prior to a meal, produced 43-55% reduction in gastric acid secretion in 11 patients.[76] In dogs, 16,16-dimethyl PGE_2 methyl ester (20) inhibited the superficial gastric lesions produced by oral aspirin and indomethacin; however, it had no effect on deep antral gastric lesions produced by repeated dosing with indomethacin.[77] 16,16-Dimethyl PGE_2 (21) given orally to dogs at 50 μg/kg did not affect gastric mucosal blood flow

under resting conditions, but significantly inhibited histamine-stimulated blood flow suggesting that its potent antisecretory effects are mediated through a direct action on gastric parietal cells.[78]

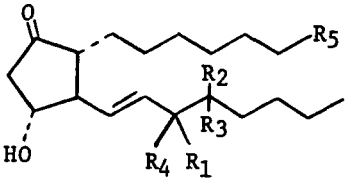
(±) 15-Deoxy-16-hydroxy-16-methyl PGE_1 methyl ester (SC 29333, 22)[79] at 0.3 and 10 µg/kg was shown to have a vasodilating action in the gastric mucosa[80] and cytoprotective effects.[81] SC 29333 is a mixture of 2 diastereomers; of this mixture, the single enantiomer 15-deoxy-(16S)-16-hydroxy-16-methyl PGE_1 methyl ester (SC 30249, 23) is believed to be the active component and is 3-9 times as active as SC 29333 as an inhibitor of gastric acid secretion. When administered to Heidenhain pouch and gastric fistula dogs at 1 µg/kg, SC 30249 completely inhibited a meal-induced gastric acid secretion but did not affect serum gastrin concentration.[82] An analog of SC 29333, 24, wherein the terminal carboxyl group is replaced by a hydroxymethylketo function, was shown to be comparable to SC 29333 in antisecretory potency and duration of effect.[83]

11-Methyl-16,16-dimethyl PGE_2 (Ro 21-6937, 25), at 10 µg/kg intravenously and 20 µg/kg intragastrically, inhibited acid output by 90% and 84%, respectively, in dogs.[84] Clinically, Ro 21-6937 was well tolerated and inhibited basal and histamine stimulated acid secretion at 7.5 µg/kg.[85]

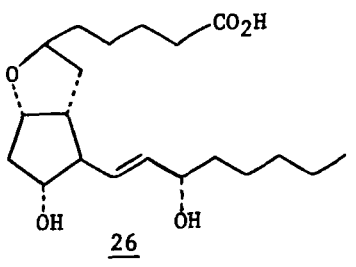
PGI_2 is the most prevalent PG in the gastrointestinal tract.[86] Both PGI_2 and PGE_2 are antisecretory (PGI_2 is 3 times more potent than PGE_2), but PGI_2 raises mucosal blood flow in the resting stomach and increases postprandial gastrin levels.[87] 5,6-Dihydroprostacyclin (6β-PGI_1, 26)[88] reduced acid output to 50% at 3.0 µg/kg intravenously, was less hypotensive than PGI_2, and elevated mucosal blood flow.[89] (5α)-5,9-Epoxy-ω-tetranor-16-phenoxy PGF_1 (27) was shown to be 2 and 4 times as potent an antisecretory agent as PGI_2 and 6β-PGI_1, respectively.[90] Carbacyclin (28) was shown to be 2-4 times less active than PGI_2 as an antisecretory agent.[91]



	R_1	R_2	R_3	R_4	R_5	R_6
19	CH_3	H	H	OH	CO_2H	H
20	H	CH_3	CH_3	OH	CO_2CH_3	H
21	H	CH_3	CH_3	OH	CO_2H	H
25	H	CH_3	CH_3	OH	CO_2H	CH_3



	R_1	R_2	R_3	R_4	R_5
22	H	CH_3	OH	H	CO_2CH_3 (11RS,16RS)
23	H	CH_3	OH	H	CO_2CH_3 (11R,16S)
24	H	CH_3	OH	H	$COCH_2OH$



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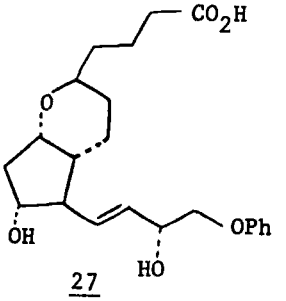

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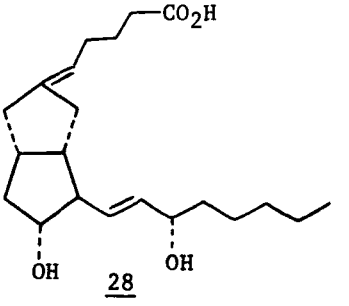


28

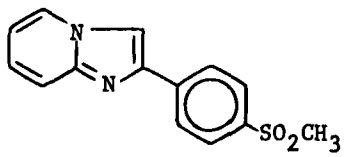
Non-prostaglandin Mucoprotectants - In a prospective multicenter trial, carbenoxolone, atropine, cimetidine and placebo were compared in patients with duodenal ulcer with no statistical differences observed.[92] An open pilot study comparing carbenoxolone and cimetidine in patients with gastric ulcer showed a slight advantage of cimetidine (86.4%) over carbenoxolone (76.5%), which was not statistically significant.[93] The mechanism of carbenoxolone's antiulcer effect is widely assumed to involve stimulation of gastric mucus synthesis; however, it is not able to counteract the observed effects of cimetidine on dissolved mucus in patients with duodenal ulcer.[94] In cimetidine-healed patients, carbenoxolone proved beneficial in maintenance therapy for prevention of ulcer recurrence.[95]

Zolimidine (29) reduced both aspirin and ethanol-induced fall in gastric transmucosal potential difference, supporting its protective effect on the gastric mucosa,[96] and significantly reduced gastric epithelial cell turnover induced by aspirin in patients with chronic gastritis.[97]

Cetraxate (DV 1006, 30)[98] given in doses of 200 and 300 mg/kg orally to rats with acetic acid-induced ulcers increased hexosamine and uronic acid content (mucopolysaccharides), which is proposed to explain the antiulcer effect.[99] Cetraxate also increases gastric mucosal blood flow.[100]

Tolimidone (31) protected rats against stress induced ulcers at 10 mg/kg orally[101] and was shown to stimulate mucus secretion.[102]

Sucralfate (32), a basic aluminum salt of sucrose sulfate, has been shown to interact with serum albumin, and thus inhibit peptic hydrolysis,[103-105] and to selectively bind to acetic acid-induced ulcers.[106-108] Sucralfate has been shown to be effective in preventing the recurrence of gastric ulcer.[109]



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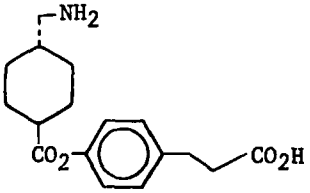

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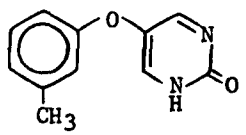

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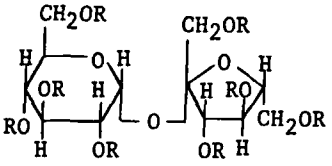


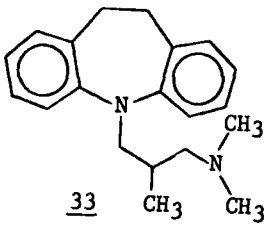
$R=SO_3\ Al_2(OH)_5$

32

CNS Active Antiulcer Agents - The tricyclic antidepressant, trimipramine (33), is being widely studied as an antiulcer agent. The impetus for this is presumably the fact that a high incidence of masked depressions has been reported in patients with duodenal ulcer.[110] In a multicenter, double-blind controlled study, patients with confirmed duodenal ulcer were treated with 50 mg of trimipramine or placebo for four weeks: 75% of trimipramine-treated patients were healed compared with 47.5% for the placebo group.[111,112] A comparable study of gastric ulcer patients showed the trimipramine-treated group to be 60% healed vs. 20% for the placebo treated group.[112,113] Both basal and pentagastrin-stimulated

acid output were reduced following administration of 50 mg of trimipramine;[114] however, no antisecretory effect was evident on insulin-stimulated secretion, suggesting a non-cholinergic mechanism. A comparative study of the effects of cimetidine, atropine, and trimipramine on histamine-stimulated gastric acid secretion in normal subjects confirmed a substantial antisecretory effect for atropine and cimetidine, and demonstrated an acid-stimulating property for trimipramine.[115] An apparent cytoprotective effect also may contribute to trimipramine's antiulcer action as shown by the drug's ability to protect against gastric mucosal barrier dysfunction induced by ethanol and hydrochloric acid in rats.[116]

The antidepressant nomifensine (34) was evaluated for its effect on stress-induced ulcers in rats; ED_{50} values for water immersion and restraint ulcers were 2.49 and 1.89 mg/kg, respectively, and there was no effect on pylorus ligation ulcers or on histamine-stimulated gastric acid secretion.[117] It was hypothesized that nomifensine's antiulcer activity was due to normalization of stress-changed catecholamine metabolism in the hypothalamic region.



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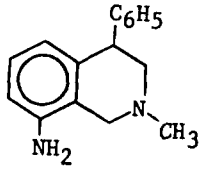

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Chapter 10. CARDIOTONIC AGENTS FOR THE TREATMENT OF HEART FAILURE

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Cardiac output is regulated by the intimate integration of the contractile state of the heart and the dynamics of the peripheral circulatory system. In heart failure, the fundamental defect is an impairment of ventricular myocardial contractility, which results in an inadequate output to meet the metabolic and circulatory demands of the body. Subsequent to depression of the contractile state of the heart, loading conditions on the heart become critical determinants of the output. Effective pharmacologic therapy of heart failure is brought about either by enhancing the contractile state of the failing pump with positive inotropic agents, or by adjustment of the peripheral circulatory state with peripheral vasodilators. The use of agents which stimulate myocardial contractility are of major value in the treatment of heart failure. Digitalis preparations have been the standard therapy for failure and are the only orally effective inotropic drugs available for the treatment of this condition. However, their cardiac electrical toxicity and peripheral vascular effects make these agents less than ideal drugs. Sympathomimetic amines are the other major class of cardiac stimulants which are used for treatment of failure. The use of these agents is limited because of oral ineffectiveness, positive chronotropic activity, arrhythmogenic properties, and unwanted peripheral vasoconstrictor actions. Presently, dobutamine (Dobutrex) and dopamine (Intropin) are the predominantly used sympathomimetic agents for heart failure. Several timely review articles have appeared which provide a comprehensive analysis of heart failure therapy with cardiotonic drugs.[1-7]

Cardiac Glycosides - Digitalis continues to be the principal pharmacologic therapy of heart failure despite the major limitations of this class of drugs. The cardiac electrical toxicity which poses significant hazards to the patient and the controversy concerning the beneficial effects of long-term therapy with digitalis[8] continue to challenge the rational use of this class of agents in heart failure. Substantial efforts have yet to clearly define the mechanism of the inotropic action, to differentiate the mechanisms of electrical toxicity and positive inotropy, and to clearly demonstrate long-term benefit with chronic use of these agents.[2-4]

Proposed models of the digitalis receptor have been suggested which consist of hydrophobic, electrostatic, and hydrogen bonding sites with the steroid, lactone, and sugar moieties of the cardiotonic steroids.[9] Using this model, the low potency and genin-like properties of actodigen have been rationalized to result from binding of the α-linked lactone ring to the receptor lactone binding site in a manner that does not allow the steroid and sugar moieties to bind as they do in digoxin.[10] Another approach uses the graphic analytical features of the NIH PROPHET computer system to model cardenolide-receptor interactions.[11] The probable manner of these specific receptor binding site(s) for cardiac glycosides has spawned a proposal for the existence of a putative endogenous substance, "endodigin," which may regulate Na,K-ATPase activity.[12]

ASI-222 (1), a semisynthetic 4-aminosugar cardiac glycoside, was compared with the neutral β-D-galactose analog, ASI-253 (2) and

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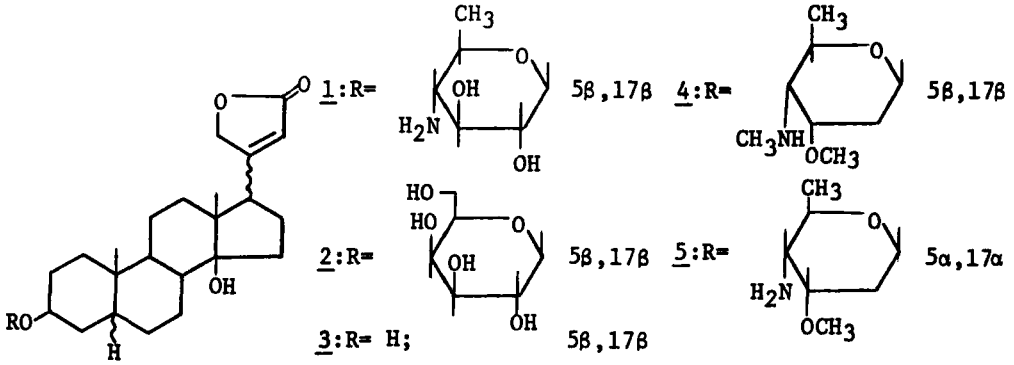
digitoxigenin (3); ASI-222 was three times more potent than 2 and two times more potent than 3 at increasing left ventricular stroke work in a dog heart-lung preparation and had a longer duration of action.[13] ASI-222 was ten times as potent as ASI-253 in inhibiting Na,K-ATPase (ID_{50} for ASI-222 was 1.3×10^{-7}M and ID_{50} for ASI-253 was 1.15×10^{-6}M).[14] The therapeutic and toxic indices (lethal dose/toxic dose) of ASI-222, ASI-253, and 3 were compared: the therapeutic indices were 2.88, 1.65, and 2.27, while the toxic indices were 1.4, 3.9, and 5.9, respectively.[15] It was suggested that more than one receptor system may account for the divergent toxic and therapeutic index ranges for the three compounds.

The electrophysiological and inotropic effects were determined for two naturally occurring aminosugar cardiac glycosides, mitiphylline (4) and holarosine B. (5).[16] In the guinea pig isolated atrium, mitiphylline produced a positive inotropic effect at concentrations from 5×10^{-9} to 5×10^{-7} g/ml, had no positive inotropic effect in the rat heart at a concentration of 5×10^{-6} g/ml, and shortened the action potential plateau in the guinea pig perfused heart. Holarosine B produced a maximal positive inotropic effect in the isolated guinea pig atrium at 5×10^{-5} g/ml and caused a negative inotropic effect in the perfused rat heart at 5×10^{-5} g/ml, which was associated with an increase in the amplitude and duration of the action potential plateau.[16]



HOE 040 (6), the 14,15-β-oxido analog of proscillaridin (7), showed one-fourth the potency of proscillaridin in producing a positive inotropic effect in the guinea pig atrium and an equi-potent positive inotropic effect in dogs, while the arrhythmogenic lethal doses were two and five times higher for HOE 040. The amount of HOE 040 absorbed from the duodenum of dogs was 72% compared with 14-25% for proscillaridin.[17]

A collection of papers concerning meproscillarin (8), the 4'-methyl ether of proscillaridin, was published, which compared the two analogs.[18] Meproscillarin is better absorbed than proscillaridin.[19] In Friedberg's-class II/III heart failure patients, a daily maintenance dose of 0.5-0.75 mg of meproscillarin induces a cumulative blood level of 1-1.5 mg after 8-10 days, which was sufficient to eliminate heart failure.[20]

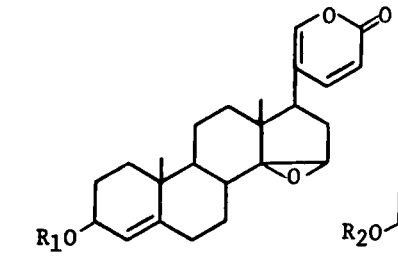
Gitoformate (9) was the subject of a symposium, the proceedings of which have been published.[21] Clinically, administration of 0.12 mg/day of gitoformate and 0.50 mg/day of digoxin to NYHA-class I/II heart failure patients resulted in similar positive inotropic responses to each drug. Unlike digoxin, gitoformate did not reduce heart rate.[22,23]

Asclepin (10) has been evaluated *in vitro* and *in vivo* in several species.[24] In cats with pentobarbital induced cardiac insufficiency, administration of 100 μg/kg of asclepin elicited a 135% increase in left

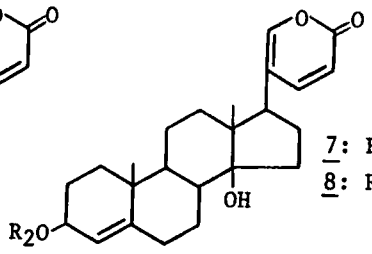
ventricular (LV) dP/dt max compared with a 79% increase with digoxin.[25]

Helveticosol-3',4'-dinitrate (11)[26] and the 3',4'-dipropionate (12) were compared: administration of 8 mg of 11 and 12 to healthy volunteers resulted in maximal shortening of systolic time intervals three hours after dosing. There was no difference between the activity of the glycosides and no perceptible cardiac or gastrointestinal side effects.[27]

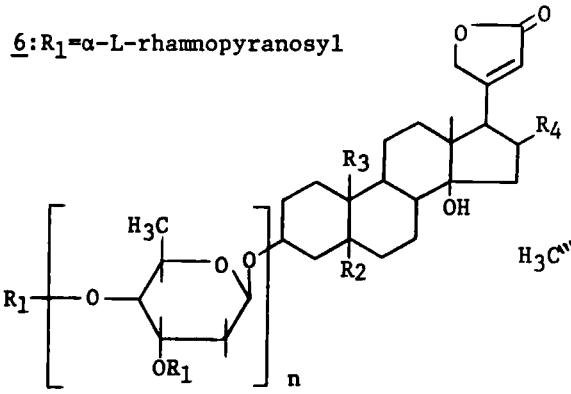
The 4-(indolin-5-yl)-2,5-dihydrofuran-2-one, 13, elicited increases in contractile force of 24 and 52% following administration of 1 and 5 mg/kg intravenously to dogs. Interestingly, the corresponding glucoside, 14, produced negative inotropic responses.[28]



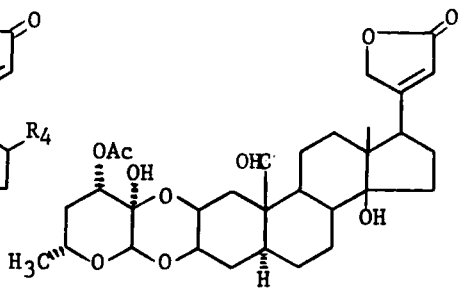
6: R_1=α-L-rhamnopyranosyl

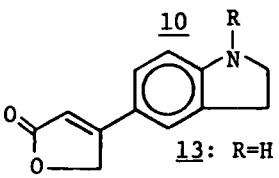


7: R_2=α-L-rhamnopyranosyl
8: R_2=4-O-methyl-α-L-rhamnopyranosyl



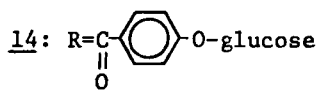
	n	R_1	R_2	R_3	R_4
9	3	CHO	H	CH_3	OCHO
11	1	NO_2	OH	CH_2OH	H
12	1	COC_3H_7	OH	CH_2OH	H





10

13: R=H



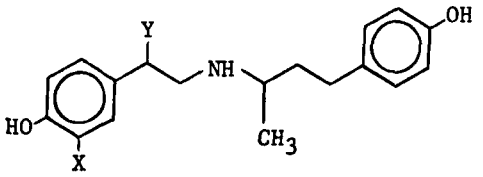
14: R=C(=O)-O-glucose

β-Adrenoceptor Stimulants - The positive inotropic action of catecholamines, such as norepinephrine, epinephrine, and isoproterenol, is accompanied by either tachycardia or vasotropic activities, which limit the use of these agents in the treatment of patients with heart failure. More recently, dopamine and dobutamine (15) have been introduced into therapy. These agents are claimed to have a more favorable profile of hemodynamic activity than the classical sympathomimetic amines. However, both dopamine and dobutamine are readily inactivated enzymatically and are orally ineffective.[29-31] These agents have been used effectively in failure associated with myocardial infarction without causing an increase in infarct size.[32] Tolerance to the hemodynamic action of dobutamine has been demonstrated in patients receiving 24 hr or four-day infusions.[31,33]

Prenalterol (H 133/22, 16) is an orally effective cardioselective β-adrenoceptor agonist.[34-38] This agent is undergoing clinical evaluation in heart failure.[39-43] Prenalterol has been shown to have a

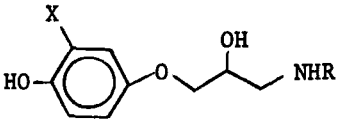
preferential positive inotropic action, as compared to its positive chronotropic action.[44,45] The cardiovascular effects of prenalterol have been favorably assessed in patients with coronary heart disease or myocardial infarction and left ventricular dysfunction.[46]

Butopamine (17) is a structural analog of dobutamine. It is not a catecholamine and is refractory to the action of catechol-O-methyltransferase. Unpublished data indicate that butopamine has a similar hemodynamic profile to that of dobutamine and that, unlike dobutamine, it is orally effective.[47] In congestive heart failure patients, intravenous administration of butopamine produced a positive inotropic effect; however, positive chronotropic activity was also noted.[48,49] In comparison to previous data from similar studies, the degree of rate increase was greater than that of dobutamine and similar to that of dopamine at equi-effective inotropic doses.[50]

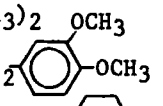


15: X=OH; Y=H

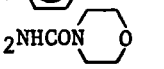
17: X=H; Y=OH



16: X=H; R=$CH(CH_3)_2$



18: X=OH; R=$(CH_2)_2$



19; X=H; R=$(CH_2)_2NHCON$

Ibopamine (SB 7505), the diisobutyric ester of N-methyldopamine, is an orally effective positive inotropic agent in anesthetized dogs and in healthy volunteers.[51] In humans, doses of 50, 100 and 150 mg of this agent caused dose-related decreases in electromechanical systole and pre-ejection period, which appeared to last for approximately 8 hrs. Insignificant changes were observed in heart rate and arterial blood pressure.

RO 363 (18) is a selective β_1-adrenoceptor agonist. In anesthetized cats, this agent had similar potency to isoproterenol as a positive chronotropic agent but was substantially less potent as a peripheral vasodilator.[52] In studies *in vitro*, RO 363 was approximately one half as potent, but equi-active, to isoproterenol as a positive chronotropic agent in guinea pig right atria and similarly potent but less active as a positive inotropic agent in guinea pig left atria and in ventricular strips from guinea pig, rat, and rabbit.[53]

ICI 118587 (19) is a new β-adrenoceptor partial agonist which has been demonstrated to possess cardioselectivity in reserpinized dogs. In these studies, a dose of 3.2 mg/kg, intravenously, was determined to be the ED_{50} for tachycardia, while at doses up to 3.0 mg/kg, ICI 118587 caused no effect on hind limb perfusion pressure.[54] Although positive inotropic action with this agent has not been reported, it is presumed that such activity is intrinsic to this compound.

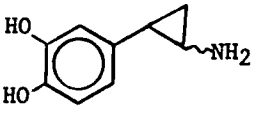
The diacetyl-L-γ-glutamyl amide of dopamine, Abbott 47884, administered intravenously to dogs produced a profile similar to subpressor doses of dopamine. A dose of 5 mg/kg produced a prolonged increase in myocardial contractility, heart rate, and renal blood flow.[55]

The dopamine analogs, (20) and (21), have been reported to produce cardiostimulatory effects similar to dopamine (the E isomer was substantially more potent than the Z isomer).[56]

A theophylline-dopamine derivative, D 4975 (22), was more active intravenously as a positive inotropic agent than dopamine or dobutamine in anesthetized dogs, and also increased heart rate and blood pressure.[57,58]

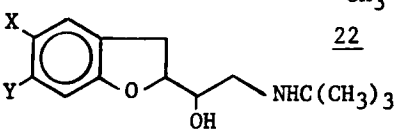
Two analogs of the β-adrenoceptor antagonist bufuralol are reported to be β-adrenoceptor agonists.[59] The 5-hydroxy analog (23) had greater affinity for vascular β-receptors while the 6-hydroxy derivative (24) had similar affinity for both receptor types.

Amezinium metilsulfate (LU-1631, 25) is a chemically and pharmacologically novel indirect-acting sympathomimetic agent.[60] This agent is an inhibitor of intraneuronal MAO and also releases norepinephrine from sympathetic nerve terminals.[61,62] This compound increased myocardial contractility, heart rate, and blood pressure in anesthetized animals.[63] In healthy human subjects, amezinium was an effective positive inotropic agent. The duration of action following intravenous administration was 90 - 120 min and was approximately 6 hrs following oral administration.[64] This compound is being considered for development as a cardiostimulant drug.[47]



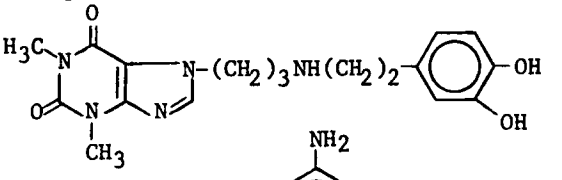
20: (E)

21: (Z)

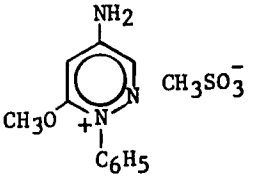


23: X=OH; Y=H

24: X=H; Y=OH



22



25

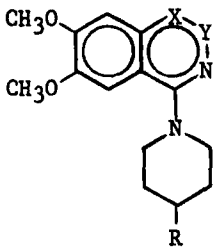
Phosphodiesterase Inhibitors - Inhibition of the degradation of cyclic AMP by phosphodiesterase (PDE) in the cardiac cell causes an increase in intracellular cyclic AMP levels. This activity has been implicated in the positive inotropic actions of several agents, although the interrelationship has not been unequivocally established.[65] Interest in this approach to the development of a cardiotonic has resulted in compounds of potential interest. Buquineran (UK 14275, 26) was previously reviewed.[66] A closely related analog, carbazeran (UK 31557, 27) is currently under development as a cardiotonic.[67,68]

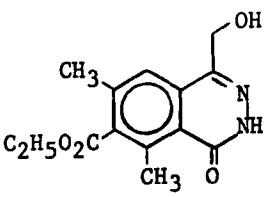
Phthalazinol (EG-626, 28) increased contractility in guinea pig atrial and ventricular muscles.[69] Although this activity was not correlated with PDE inhibitory activity, this relationship was suggested based on previous data.[70]

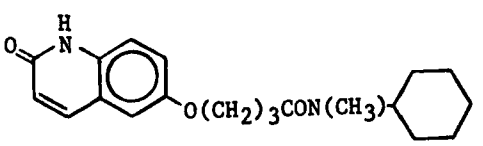
Cilostamide (OPC-3689, 29), an antithrombotic agent which inhibits PDE,[71] increased myocardial contractility in rabbit and dog hearts. This agent was more potent but less active than IBMX in rabbit ventricular muscle, whereas cilostamide and IBMX had comparable activities in dog ventricular muscle. This difference was ascribed to species differences in basal turnover rates of cyclic AMP.[72]

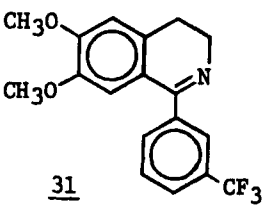
Recent studies indicate that the PDE inhibitor AP10 (30) has inotropic activity and antiarrhythmic activity.[73]

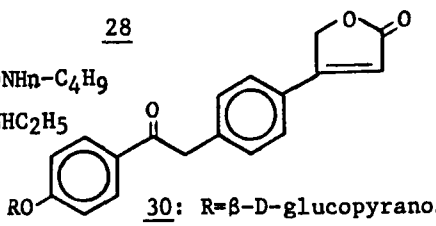
USV 2776 (31) and related dihydro- and tetrahydro-isoquinolines inhibit cardiac cyclic nucleotide PDE, although no demonstration was made of positive inotropic action.[74]











26: X=N; Y=CH; R=NHCONHn-C_4H_9

27: X=CH; Y=N; R=$OCONHC_2H_5$

28

29

30: R=β-D-glucopyranosyl

31

Novel Cardiotonics - One of the most promising positive inotropic agents to be studied in recent years is the bipyridyl analog amrinone (Win 40680, 32).[75,76] In isolated cat atria and papillary muscles, amrinone (3-1000 μg/ml) caused significant dose-related increases in developed tension with relatively smaller changes in right atrial rate.[77] In the anesthetized dog, a 1 mg/kg intravenous dose of amrinone caused a 75% peak increase in cardiac contractile force measured by a strain gauge and a 60% peak increase in LV dP/dt max. Administration of amrinone orally at 10 mg/kg caused a 57% increase in LV dP/dt max with an onset of 45 minutes and a total duration of the inotropic effect of more than 5 hours.[77] In pentobarbital-induced heart failure in dogs, amrinone caused an increase in both contractile force and cardiac output. In isolated blood vessel strips[78] and in the canine isolated perfused hindlimb,[79] a direct coronary vasodilation distinct from that occurring secondary to inotropic activity was demonstrated for amrinone.

The mechanism of amrinone's inotropic action has been the subject of intense investigation. It is not a direct or indirect β-adrenoceptor agonist, does not increase cyclic-AMP, does not inhibit Na,K-ATPase, and does not stimulate histamine receptors.[76,77] Amrinone increased contractility and stimulated influx of taurine into the guinea pig but not the rat heart.[80] Studies using the calcium sensitive protein aequorin[81] and dog erythrocytes[82] suggest that amrinone increases the transport of calcium across the sarcolemma, thus increasing intracellular calcium-levels. Other studies show that amrinone is a nonspecific relaxant of smooth muscle, acting at multiple sites to decrease the availability of calcium which may account for its vasodilator properties.[83]

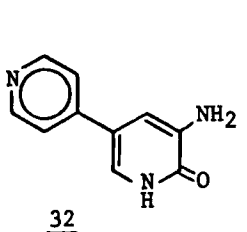
Several clinical studies in heart failure showed that in patients receiving concommitant doses of digitalis, amrinone elicited increases in cardiac output, stroke volume, stroke work, and LV dP/dt max, while capillary wedge pressure declined. Heart rate remained unchanged, while myocardial oxygen consumption decreased.[84-87] Two of nine patients receiving amrinone developed severe but reversible thrombocytopenia.[88] Experiments in dogs with experimentally induced ischemia suggest that amrinone and isoproterenol are equally detrimental in increasing acute ischemia and myocardial injury, which may limit the potential use of amrinone in heart failure patients with acute myocardial ischemia.[89] Peak plasma concentrations of drug occurred between 0.25-3 hours. The mean $t_{1/2}$ was 2.94 ± .21 hours suggesting an oral dosage regimen of 4-6 hours.[90] The development and pharmacological activity of amrinone has been reviewed.[91]

The carbazole derivative, Win 35020 (33), was shown to be a positive inotropic agent both *in vitro* and *in vivo*, causing a dose-related increase in contractile force at 3-100 µg/ml in the isolated cat papillary muscle preparation, at 0.1-3.0 mg/kg intravenously in the anesthetized dog, and at 15-60 mg/kg orally in conscious dogs.[76] Pretreatment with reserpine resulted in complete elimination of the inotropic response *in vitro* and a diminution of the response *in vivo* indicating that the mechanism of action was due to release of norepinephrine.[76] Hemolytic anemia appeared in dogs following oral administration of 33 at 50 mg/kg for 50 days.

AR-L 57 (34) and AR-L 115 (35) are novel positive inotropic agents with unknown mechanisms of action. In normal human males, a single intravenous dose of 200 mg of AR-L 57 caused a significant inotropic response of short duration, suggesting utility in the intensive care facility.[92] In squid axons, AR-L 57 rapidly and reversibly inhibited the sodium pump in some ways resembling the action of cardiac glycosides; the action was not dependent upon external sodium ions, suggesting a difference in the molecular mode of interaction with the sodium pump.[93] AR-L 115 displayed positive inotropic effects *in vitro* at 10-300 µM and increased LV dP/dt max 12-123% at 0.1-2.0 mg/kg intravenously in the anesthetized dog. Orally, in conscious dogs, AR-L 115 increased LV dp/dt max by 16-92% for 5-12 hours following administration of 2.5-30 mg/kg.[94] β-adrenoceptor blockade did not diminish the inotropic effect of AR-L 115 and the therapeutic ratio was shown to be 17 times that of ouabain.[95] In patients with NYHA-class III/IV heart failure, AR-L 115 at 3 mg/kg intravenously produced increases in heart rate (18%) cardiac output (21%) and a decrease in arterial pressure (25%). At 200 mg orally t.i.d., AR-L 115 improved cardiac output (31%) and ejection fraction (35%) in patients with congestive cardiomyopathy. The agent did not cause an enhancement of ectopic activity in the heart and did not cause conduction defects.[96]

RMI 82,249 (36), 0.3-10.0 mg/kg intravenously, increased myocardial contractile force and heart rate, and decreased blood pressure in anesthetized dogs. These effects were not blocked by propranolol.[97] RMI 82,249 produced greater hemodynamic effects in dogs with experimentally-induced heart failure than in normal dogs.[98, 99]

The antiarrhythmic agent, BIIA, 37, produced significant positive inotropic effects *in vitro* at 0.3-10.0 µmol/ℓ and *in vivo* in the anesthetized cat at 2.0 mg/kg intravenously. Inotropic action is not mediated by α- or β-adrenoceptors. In the guinea pig heart and kidney BIIA inhibits Na,K-ATPase, but unlike the cardiac glycosides this inhibition is competitive with sodium.[100]



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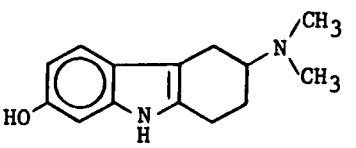

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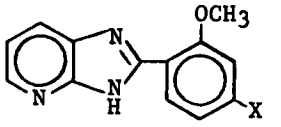
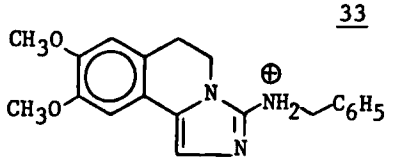
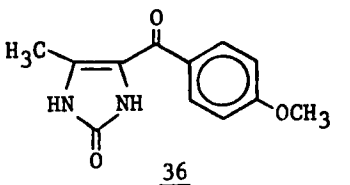


34: X=OCH_3

35: X=$SOCH_3$



37 $Cl^{\ominus}$



36

Ionophores - The ionophore monensin, which preferentially complexes sodium, has undergone study as a cardiotonic agent. In anesthetized dogs, monensin at doses of 5-25 μg/kg produced dose-dependent increases in coronary blood flow and decreased total peripheral resistance, while at doses greater than 25 μg/kg it increased myocardial contractility and aortic blood pressure.[102] The combination of low concentrations of ouabain with monensin led to irreversible contracture and irregular responses to electrical stimulation in isolated rabbit left atria.[103] In anesthetized dogs, the potassium complexing ionophores grisorixin (2 mg/kg) and alborixin (1 mg/kg) produced 250 and 320% increases in ventricular contractile force accompanied by ECG disorders similar to those described for hypokalemia.[104,105] In the isolated working rat heart preparation, the calcium complexing ionophore X-537A (Ro-2-2985) increased heart rate and contractility. The increase in heart rate was eliminated by reserpine pretreatment or β-adrenoceptor blockade, while the increase in contractility was only partially attenuated, indicating a direct inotropic effect of the ionophore.[106]

Peptides - Three peptides with similar cardiotonic activity have been isolated from the sea anemone: Anthopleuran-A (AP-A) and AP-C have been sequenced, while AP-B has been partially sequenced.[107] *In vitro*, AP-A increased force of contractions of isolated cat heart papillary muscles at doses as low as 2×10^{-9}M.[108] Dantrolene inhibits the positive inotropic effect of AP-A, suggesting that the mechanism of action may be an intracellular translocation of calcium, unlike ouabain or isoproterenol which require increased transmembrane calcium flux.[109]

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Chapter 11. Renal Blood Flow and Dopaminergic Agonists

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Introduction - The afferent and efferent arterioles are the major sites of renal vascular resistance. Changes in the resistance within either of these vessels will alter the renal blood flow (RBF). Differential changes in the resistances of both vessels determine the hydrostatic pressure in the glomerular capillaries and, therefore, the glomerular filtration rate (GFR).[1] A net decrease in perfusion resistance proximal to the glomerulus effects a concurrent increase in RBF and GFR.[2]

Vasodilator substances increase RBF and augment sodium excretion. The exact mechanism of this natriuresis is not fully delineated but may be due to hemodynamic changes[3] or direct tubular effects.[4,5]

The phenomenon of autoregulation tends to maintain constant RBF and GFR in a pressure range of 80 to 180 mm Hg[6,7] and may be mediated by prostaglandins.[8-10] However, sympathetic input[11] and numerous humoral agents including catecholamines,[12] ADH,[6] angiotensin,[13,14] prostaglandins,[15] histamine[16] and bradykinin[17] can greatly alter renal hemodynamics. Since other vascular beds respond to most of these stimuli as well, the selective alteration of renal hemodynamics is difficult.

The presence of specific postsynaptic dopaminergic receptors in the kidney distinguishes it from most other vascular beds.[18] These postsynaptic receptors on stimulation directly induce renal vasodilation.[19] Structurally distinct presynaptic dopaminergic receptors present in peripheral sympathetic nerve endings attenuate norepinephrine release.[20]

Dopamine and Adrenergic Receptors - Dopamine exerts complex effects on the cardiovascular system, which are mediated by a combination of alpha adrenergic, beta adrenergic and peripheral vascular dopaminergic receptor stimulation.[19,21] The existence of vascular dopamine receptors has been generally accepted since the *in vivo* demonstration of a specific vasodilator effect of dopamine.[21,22] These receptors occur principally in the renal and mesenteric vessels. Notably, the dose of dopamine required to produce renal vasodilation is approximately one-tenth that necessary to elicit pressor, inotropic and chronotropic effects due to alpha and beta adrenergic stimulation.[18,24-26] In addition to its action on renal blood flow, dopamine also produces a diuresis and natriuresis in rats, dogs and humans with little effect on potassium excretion.[18,27-30] The specific action of dopamine at low doses on the renal vasculature suggests its use for the treatment of cardiovascular disease. However, since dopamine is not absorbed on oral dosing, several orally active prodrugs and dopaminergic agonists have been developed.

Dopaminergic Prodrugs - Ibopamine (SB 7505, 1), the diisobutyric ester of epinine, is an orally active renal vasodilator and diuretic in

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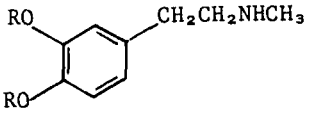
animals and man.[31,32] Urinary excretion of sodium and potassium increased significantly, but the Na^+/K^+ ratio did not change as compared to the baseline values (placebo).[33] In addition, 1 potentiates furosemide diuresis,[34] and is devoid of systemic pressor effects and significant CNS activity (see Chapter 10).[31,32,34]

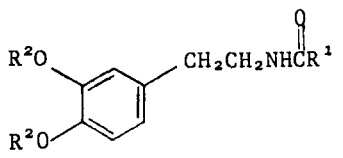
The isobutyryl moieties of 1 facilitate oral absorption. Slow cleavage of these groups by serum esterases releases quantities of epinine (2), a compound equipotent to dopamine as a renal vasodilator.[18]



1 R=CCH(CH₃)₂

2 R=H

Orally active prodrugs have also been produced by the acylation of the phenethylamine side chain of dopamine with amino acid residues.[35-38] Unlike the alanyl (Abbott 37301, 3) and leucyl (Abbott 41596, 4) analogs, the γ-glutamyl dopamine analog (5) is not a substrate for ubiquitous serum aminoacylarylamidases and is therefore devoid of systemic effects.[39,40] The glutamyl residue is specifically removed by γ-glutamyl transpeptidase, an enzyme which has its highest concentration in the kidney.[41-43] On passage through the kidneys, 5 releases dopamine which induces renal vasodilation but does not re-enter the systemic circulation.[39,40] Intravenous administration of 6 (Abbott 47884) to dogs produces a profile similar to subpressor doses of dopamine. A dose of 5 mg/kg elicits a prolonged increase in RBF, myocardial contractility and heart rate.[44]



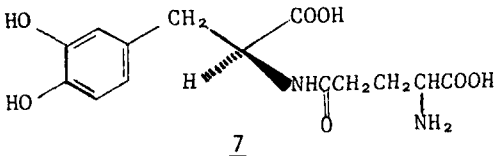
3 R^1=CH(NH₂)CH₃, R^2=H

4 R^1=CH(NH₂)CH₂CH(CH₃)₂, R^2=H

5 R^1=CH₂CH₂CH(NH₂)CO₂CH₂Ø, R^2=H

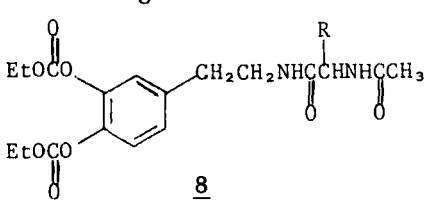
6 R^1=CH₂CH₂CH(NH₂)COOH, R^2=CH₃C(=O)

Selective accumulation of dopamine in the kidney after oral administration of 7 has also been reported.[45] Di- and tripeptide analogs of L-DOPA are also specific dopaminergic renal vasodilators devoid of systemic effects.[46]



7

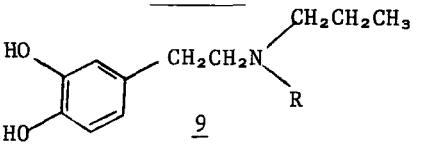
Related compounds in which the catechol moiety is protected as a bis-carbonate, e.g. 8, are long acting, orally effective, renal vasodilators having no effect on heart rate (HR) or blood pressure (BP).[47]



8 R=CH₃ or -CH₂CH₂SCH₃

N-Alkyl Dopamine Analogs - In addition to dopamine (or epinine) prodrugs, several alkyl analogs of dopamine have been prepared with the hope of increasing the renal dopaminergic effects and attenuating the adrenergic effects.[48,49] In general, N-alkyl analogs of dopamine or epinine (2) are inactive as renal vasodilators, or their effects are blocked by propranolol indicating β-stimulation. N,N-Dialkyl analogs of dopamine, in which one alkyl moiety is n-propyl, cause renal vasodilation, which is blocked by haloperidol but not propranolol. The relative potency of some of these analogs (9) is shown in Table I. With the exception of 9c, symmetrically substituted dopamine analogs are inactive,[50,51] as are compounds 9g and 9h.

Table I

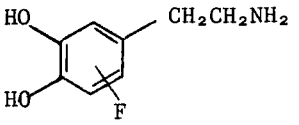


9

	Relative Potency		Relative Potency
dopamine	100		
a R = n-butyl	5	e R = phenethyl	0.7
b R = n-pentyl	4	f R = ethyl	0.6
c R = n-propyl	3	g R = s-butyl	--
d R = i-butyl	0.7	h R = benzyl	--

In a homologous series of dopamine analogs, the N-n-propyl substituent is a requirement for optimum dopaminergic activity. Even though the compounds in Table I are 10-100 times less potent than dopamine as renal vasodilators, they lack its β-adrenergic activity but retain α-adrenergic effects.[49] Acylation of the catechol moiety of 9 makes these agents orally active.[48]

Miscellaneous Dopamine Analogs - Modification of the catechol nucleus of dopamine usually results in inactive compounds.[52] However, fluorine substitution on the aromatic ring caused only minor changes in dopaminergic activity. Compounds 10 and 11 are essentially equipotent to dopamine as renal vasodilators. The 6-fluoro isomer (12) is about four times less potent. Unfortunately, adrenergic effects were not altered.[53]

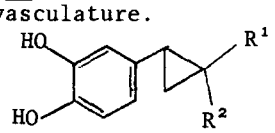


10 2-fluoro

11 5-fluoro

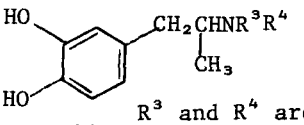
12 6-fluoro

Alkyl substitution on the phenethylamine side chain of dopamine also diminishes renal dopaminergic activity.[52] The phenylcyclopropylamines (13)[54] and α-methyl dopamine analogs (14)[55] do not dilate the renal vasculature.



13 a R^1=H, R^2=NH_2

b R^1=NH_2, R^2=H

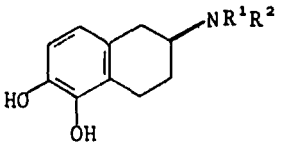


14

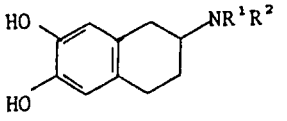
R^3 and R^4 are combinations of H, $-CH_3$, $-CH_2CH_3$, $-CH_2CH_2CH_3$, $-CH(CH_3)_2$, and $-CH_2CH_2CH_2CH_3$.

Aminotetralins - In addition to dopamine analogs, several types of bicyclic systems have been tested as renal vasodilators. The dihydroxy-2-aminotetralins 15 and 16 represent semi-rigid analogs of the α- and β-rotamers of dopamine. Although an effective dopaminergic agonist in the CNS, 5,6-ADTN (15a) is inactive in the kidney. Surprisingly, the N,N-di-n-propyl-5,6-ADTN (15b) is equipotent to dopamine as a renal vasodilator.[56] In contrast to 15a, 6,7-ADTN (16a) is a potent renal dopaminergic.[57,58] The N-alkyl analogs of 16, including the di-n-propyl compound (16c), are not as active as 6,7-ADTN (see Table II). Comparison of the R and S enantiomers of 16a indicates the R form is the active isomer.[59]

Table II



15



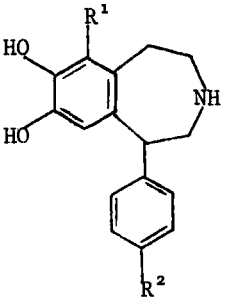
16

	R^1	R^2	Relative Potency
dopamine	--	--	100
15a	H	H	inactive
15b	$-CH_2CH_2CH_3$	$-CH_2CH_2CH_3$	100
16a	H	H	100
16b	CH_3	H	~100
16c	$-CH_2CH_2CH_3$	$-CH_2CH_2CH_3$	25
16d	$-CH_2CH_2CH_2CH_3$	$-CH_2CH_2CH_3$	25

Notably, 16a does not cross the blood-brain barrier and can be made orally active by acylation of the catechol hydroxy groups.[60]

The activity of 15b clearly demonstrates the need for further work to fully define the structural requirements of the renal dopaminergic receptor. It is clear that there is no strict requirement for the α- or β-rotameric configuration.

Benzazepines - A novel series of dopaminergic agonists has been developed. The parent compound 17 (SKF 38393) is about one-tenth dopamine as a renal vasodilator in the anesthetized dog. When administered ip, 17 causes diuresis and natriuresis in rats.[61,62] Although it is long acting and has minimal adrenergic effects,[61,62] 17 produces submaximal decreases in renal vascular resistance (RVR) and increases in RBF compared to dopamine. The renal depressor effects of 17 and dopamine are additive.



17 $R^1=R^2=H$ (SKF 38393)

18 $R^1=Cl$, $R^2=OH$ (SKF 82526)

19 $R^1=Cl$, $R^2=H$

20 $R^1=H$, $R^2=OH$

Since preliminary data indicates that a portion of the renal vasodilation produced by dopamine is due to presynaptic dopaminergic sympathetic attenuation,[63] 17 may be a selective postsynaptic dopaminergic agonist.[61,62]

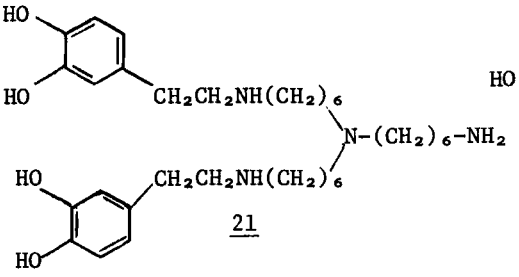
Interestingly, 18 (SKF 82526) is nine times more potent than dopamine and one hundred times more potent than 17 as a renal vasodilator in the dog.[64] The maximal vasodilation obtained with 18 is 50% greater than that produced by dopamine. When administered parenterally, 18 is a potent, kidney selective, adenylate cyclase mediated dopaminergic agonist, which does not cross the blood-brain barrier. Unlike 16 and dopamine, 18 has little effect on HR, and at higher doses it produces modest hypotension.[64]

The des-hydroxy analog (19) is equivalent to dopamine in the renal vasculature both in potency and in maximal decrease in RVR. The des-chloro analog (20) is inactive as a renal depressor.[64]

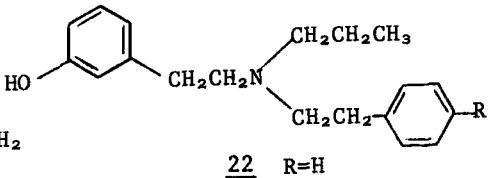
The effects of these analogs (17-20) are antagonized by haloperidol, chlorpromazine and bulbocapnine.

Miscellaneous Dopaminergic Agonists - The dimeric agonist 21 (Sandoz 27403) lowers blood pressure at 2.5 mg/kg i.v. in dogs by dilation of the renal and mesenteric vascular beds, but the compound is not orally active. The hypotensive action of 21 is blocked by haloperidol.[65]

Compounds 22 (RU 24213) and 23 (RU 24926) are orally active dopaminergic agonists related to the N,N-dialkyldopamine analogs discussed above. These compounds are potent dopaminergic agonists at the straital and anterior pituitary levels in rats.[67]

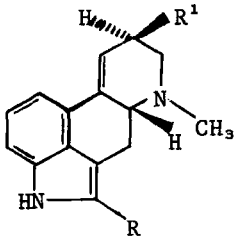


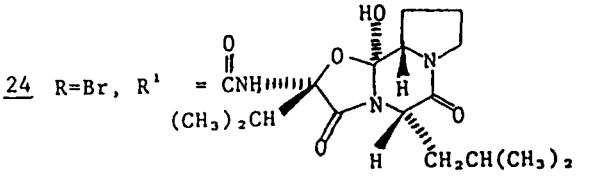
21



22 R=H

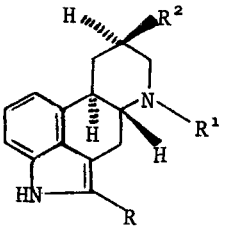
23 R=OH





24 R=Br, R^1 =

25 R=H, R^1=NHCN$(CH_2CH_3)_2$



26 R=Cl; R^1=CH_3; R^2=CH_2CN

27 R=H; R^1=$CH_2CH_2CH_3$; R^2=CH_2SCH_3

28 R=H; R^1=CH_3; R^2=CH_2S-

Analogs of the ergot alkaloids have been studied as dopaminergic agonists.[68,69] Although these alkaloids exhibit a broad range of pharmacological activities,[70] some analogs are effective dopaminergic agents, e.g. bromocriptine (24),[71-75] lisuride (25), lergotrile (26),[76-78] pergolide (27)[78-80] and CF 25397 (28).[75] Although primarily utilized for CNS disorders, these compounds also lower BP and HR in the SH rat -- effects blocked by haloperidol. In the case of 26 and 27, these actions are due to stimulation of presynaptic dopaminergic receptors; they are inactive in the renal vasculature.[81,82] Bromocriptine (24), though, is one-tenth dopamine as a renal vasodilator. This depressor effect persists up to 40 minutes following termination of the infusion of 24.[71-75]

Renal Dopaminergic Receptors - The structural requirements for agonists at the postsynaptic renal receptor are quite specific and have been discussed by several authors. Although not fully defined, some general aspects of this receptor can be discussed. Until recently, the β-rotamer of dopamine appeared to be the preferred configuration.[83] However, recent studies with 15b[56] suggest the rotameric configuration of dopaminergic agonists at this receptor may be of secondary importance. Likewise, the proposed necessity of an antiperiplanar arrangement of the catechol ring and amine moieties[84-87] is not consistent with renal vasodilator activity for the benzazepines 17 and 18.[61,62,64] A receptor model simply requiring a planar arrangement of the catechol ring and amine moieties separated by 7-8 Å with no requirement for a specific rotameric configuration or trans geometry was recently proposed.[88] A steric limitation 2 Å above the plane was included to explain the inactivity of dopamine analogs having alkyl groups on the phenethyl sidechain, e.g. 13 and 14.[88] A hydrophobic binding site may also be present in the plane to accommodate the phenyl ring of 17 and 18.[88]

The addition to this receptor model of a sterically specific hydrophobic binding area adjacent to the amine moiety will help explain the observed effects of N-n-propyl substituents.[49] Likewise, the 100-fold potency difference between 17 and 18 may require the inclusion of additional hydrophobic and hydrogen bonding sites to accommodate the chloro and hydroxy substituents of 18.[64] Presently, receptor models still fail to explain why only the R enantiomer of 16a is a renal vasodilator.[59]

POTENTIAL CLINICAL INDICATIONS

Shock - Dopamine is widely used in the treatment of shock because of its ability to increase cardiac output by action on beta$_1$-adrenergic receptors, to increase renal and mesenteric blood flow by action on postsynaptic dopamine receptors, and to increase arterial blood pressure by action on alpha-adrenergic receptors.[19-26] Activation of these receptors is dose-related. At lower doses renal blood flow increases; when the dose is increased, cardiac output increases. Finally, vasoconstriction occurs. Excessive vasoconstriction is one of the adverse effects of dopamine, and thus, compounds with reduced alpha-adrenergic activity would have application in the treatment of shock. At the present time, alpha-adrenergic blocking agents or non-specific vasodilating agents, such as nitroprusside or nitroglycerin, are infused concomitantly with dopamine when it is necessary to reduce vasoconstriction.[24-26]

Congestive Heart Failure - Dopamine has been used successfully in the acute treatment of refractory heart failure.[19-26] Increase in cardiac output, renal blood flow, and sodium excretion frequently occurs when dopamine is injected intravenously in these patients. Use of dopamine in the treatment of congestive heart failure, however, is limited by its alpha-adrenergic vasoconstricting effect, which increases the workload of the heart, and by its poor oral efficacy. In acute treatment of congestive heart failure, the infusion rate of dopamine is adjusted to avoid vasoconstriction. Alternatively, vasodilating agents, such as nitroprusside and nitroglycerin, are added to reduce vasoconstriction.[24-26] Development of an oral dopamine derivative active only on $beta_1$ and postsynaptic dopaminergic receptors could have extensive use in the treatment of chronic congestive heart failure. Limited studies with levodopa (which is decarboxylated and continuously generates dopamine for a period of about 30 min) have shown that the concept is practical.[89,90] Ibopamine (1) should also be effective in treating congestive heart failure. Inclusion of presynaptic dopaminergic receptor activity in a compound also might be desirable, since such an action would reduce the excessive sympathetic nervous system activity present in these patients. Intravenous administration of N-propyl-N-butyl dopamine (9a) produced hemodynamic improvement in a small number of patients with congestive heart failure.[91-93] Since this compound does not act on $beta_1$-adrenergic receptors,[50,51] the improvement seen in these patients appears to have been the result of the action of this compound on presynaptic dopaminergic receptors.

Hypertension - Administration of dopamine in the presence of an alpha-adrenergic blocking agent markedly reduces blood pressure in hypertensive patients, presumably by its vasodilating actions.[19,21] Accordingly, a compound which acts only on postsynaptic dopaminergic receptors should lower BP and maintain RBF in hypertensive patients. Since most antihypertensive agents do not increase RBF, development of an orally effective dopaminergic agonist without alpha- and beta-adrenergic receptor actions should be an important contribution to the treatment of hypertension. Compounds which act on presynaptic dopaminergic receptors also have antihypertensive efficacy,[71-75] and it is possible that new drugs which combine postsynaptic and presynaptic dopaminergic receptor agonist activity may have an advantage over compounds acting on a single dopaminergic receptor.

Renal failure - Reduction of renal perfusion is frequently followed by renal failure. Agents which improve RBF have potential therapeutic indications for prevention and treatment of acute renal failure. Dopamine markedly improves renal function in hypotensive patients and in patients with cirrhosis.[19,21,24-26] Intravenous infusion of dopamine in low concentrations has been shown to promote diuresis in patients with early acute tubular necrosis, and there are a few reports of beneficial effects of dopamine in patients with chronic renal failure. Preliminary studies with ibopamine (1) also have shown beneficial effects in a few patients. These studies clearly indicate the need for additional orally active dopaminergic agonists in patients with renal failure.

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Section III - Chemotherapeutic Agents

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Ann Arbor, MI 48105

Chapter 12. Antibacterial Agents

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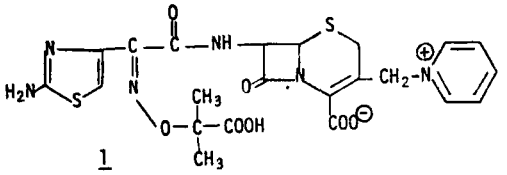
Introduction - The purpose of this chapter is to update and acquaint the reader with the newer antibacterial agents. However, topics describing the biosynthesis of these agents will not be included. A number of commmunications relating to antibacterial agents were recently presented at the 20th Interscience Conference on Antimicrobial Agents and Chemotherapy and the abstracts published.[1] In addition, there are recent comprehensive reviews regarding the origin, properties and biosynthesis of antibiotics.[2,3]

Cephalosporins and Related Analogs - Moxalactam (LY127935),[4,5] cefotaxime (HR-756)[6,7] and cefoperazone (T-1551)[8,9] continue to undergo intensive clinical trials. Extensive laboratory and clinical evaluations also are ongoing with ceftizoxime (FK-749)[10-12] and ceforanide (BL S786).[13,14] Both of these antibiotics display excellent pharmacokinetics.[15-17] There is continued interest in the development of broad spectrum, long-acting β-lactamase resistant cephalosporins with increased antipseudomonal activity. Ceftazidine (GR 20263, 1), a new broad spectrum cephalosporin, has activity



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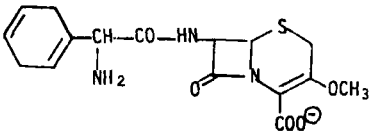

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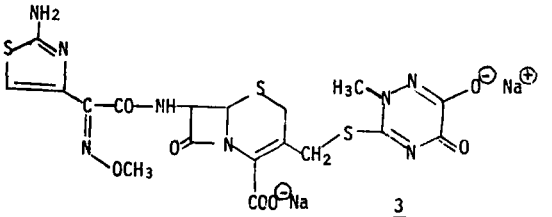

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against Pseudomonas aeruginosa similar to that of tobramycin.[18] It is stable to a variety of β-lactamases[19], is safe in humans and has an excellent pharmacokinetic profile.[20] AC-1370 is a parenterally active broad spectrum cephalosporin, which is 15 times more active than carbenicillin against P. aeruginosa. It also is stable to a variety of cephalosporinases.[21] SM-1652 is another new long-acting broad spectrum antipseudomonal antibiotic.[22] The orally active cephalosporin, cefroxadine (CGP-9000, 2), has an antibacterial

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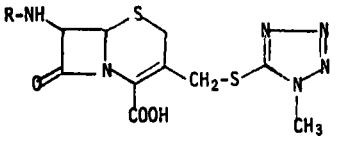
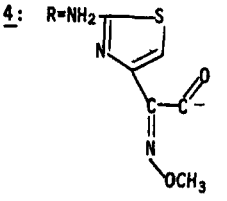
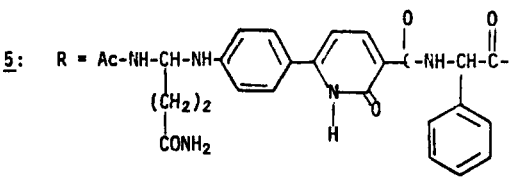
spectrum and pharmacokinetic profile similar to cephalexin.[23,24] RO-13-9904 (3) is a new long-acting semi-synthetic parenteral compound with an overall antibacterial spectrum similar to the other new third generation parenteral cephalosporins.[25,26] This antibiotic has excellent activity against both non-penicillinase producing and penicillinase producing Neisseria gonorrhoeae.[27] In vitro and in vivo data also have been reported on two other new broad spectrum cephalosporins, cefmenoxime (SCE-1365, 4)[28-31] and CN-92,982 (5).[32] Studies on new 1-oxa-cephalosporin derivatives continue.[33] Work also continues on YM-09330, a long-acting β-lactamase stable cephamycin-type antibiotic.[34,35] MT-141 is a new cephamycin, which has an antibacterial spectrum similar to cefmetazole

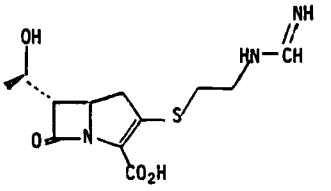






and cefoxitin. However, it does have better activity against anaerobic bacteria,[36] a longer half-life and a shorter killing time than cefmetazole and cefoxitin.[37] In vivo studies are ongoing with cefmetazole.[38]

Penicillins and Other β-Lactams - The total synthesis of thienamycin was reported.[39-41] Considerable in vitro and in vivo studies continue on the N-formimidoyl derivative (MK 0787, 6) of thienamycin.



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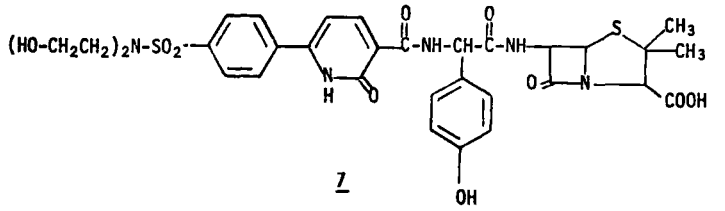
MK 0787 is extremely active against most gram-positive and gram-negative organisms, including Acinetobacter, Serratia, Pseudomonas and Bacteroides.[42-48] However, this compound is readily metabolized by the renal enzyme dehydropeptidase-I, and the co-administration of the reversible enzyme inhibitor, 3-substituted-Z-2(S-2,2-dimethylcyclopropylcarboxamido) propenoate, may be necessary to elevate urine levels.[49-51] EMO 39 734 is a new broad spectrum pyridylureidopenicillin, which is more active against P. aeruginosa than gentamicin.[52] Another penicillin with antipseudomonal activity is CI-867 (7). However, it is not active against penicillin G resistant Staphylococcus aureus, and its activity is considerably reduced when the inoculum is increased.[53]



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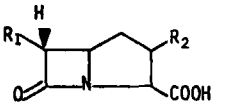
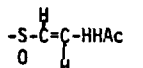
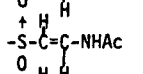
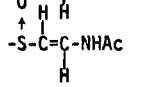
The structures and antibacterial activities of a series of new β-lactams (PS-5, 8a, PS-6, 8b and PS-7, 8c) belonging to the penem family were reported.[54-56] A new class of carbapenem antibiotics was reported, and it is represented by carpetimycin A (9a) and B (9b), which are identical to C-19393H2 and C-19393S2, respectively. The activity of (9b) was shown to be 4-50 times that of cefoxitin.[57,58]

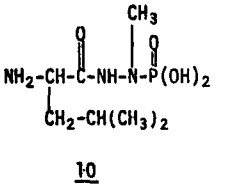


	R_1	R_2
8a:	C_2H_5-	-S-$(CH_2)_2$NHAc
8b:	1-C_3H_7-	-S-$(CH_2)_2$NHAc
8c:	C_2H_5	
9a:	$(CH_3)_2$COH	
9b:	$(CH_3)_2$C-OSO_3H	

β-Lactamase Inhibitors - Studies continue on β-lactamase inhibitors. Significant differences in antibacterial activity and β-lactamase inhibition were reported between several olivanic acid derivatives (MM4550, MM13902, MM17880, MM22380, MM22381, MM22382 and MM22383) and the related antibiotics N-acetylthienamycin and thienamycin. These studies included structure-activity relationships.[59] CP-45,899, a semi-synthetic penicillanic acid, was shown to potentiate the activity of ampicillin and other β-lactams against β-lactamase producing organisms.[60] However, while more stable and less toxic than clavulanic acid,[61] CP-45,899 is less potent than clavulanic acid[62] and is only sporadically synergistic with β-lactams against multi-drug resistant Enterobacteriaceae and Pseudomonas.[63] An oral formulation of amoxicillin and clavulanic acid (Augmentin®, BRL-25,000) was reported to show good clinical efficacy in the treatment of soft tissue[64,65] and upper and lower urinary tract infections.[66] Augmentin also demonstrated good activity against experimentally induced Bacteroides fragilis[9] and ampicillin resistant Haemophilus influenzae infections.[67] Penicillanic acid sulfone was reported to act synergistically with mecillinam, cefoperazone, piperacillin and cefoxitin against a large number of gram-positive, gram-negative and anerobic bacteria.[68-71] A series of 6-β-halopenicillanic acids (6- β-bromo, 6-β-chloro and 6-β-iodo) has been obtained and their structures and β-lactamase activities described.[72] Ceftizoxime (FK 749) was found to inhibit and display competitive inhibition kinetics against Type I β-lactamases.[73]

Cell Wall Inhibitors - Interest in screening for new cell wall inhibitors has resulted in the discovery of FR-900137 (10), a new phosphorus containing antibiotic produced by Streptomyces unzenensis. This agent inhibits the growth of a wide spectrum of gram-positive and

gram-negative bacteria, except P. aeruginosa.[74,75] The chemical structure and synthesis of phosphonic acid antibiotics FR-90098 (14) and its N-formyl analog, FR-31564 (13), has been described, as well as that of FR-33289 (11) and FR-32863 (12).[76,77] Comparative studies against gram-positive bacteria, P. aeruginosa and various Enterobacteriaceae showed FR-31564 inhibited strains resistant to ampicillin, gentamicin and fosfomycin in vitro and protected mice from experimental E. coli and P. aeruginosa infections by the oral or intramuscular route.[78] The human pharmacokinetics of FR-31564 have been reported with no adverse effects.[79]



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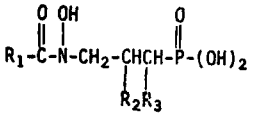


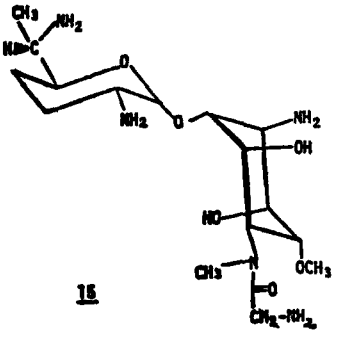
11: $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$

12: $R_1 = H$, R_2, R_3 = olefinic bond

13: $R_1 = CH_3$, $R_2 = R_3 = H$

14: $R_1 = H$, $R_2 = R_3 = H$

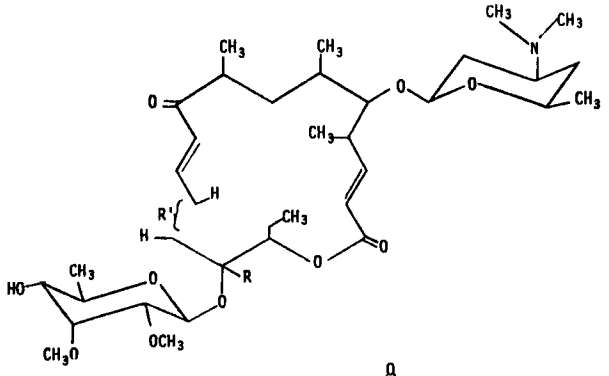
Aminoglycosides - There has been considerable interest in the antimicrobial properties of fortimicin A (KW 1070, 15). Recent studies reveal that (15) is similar to kanamycin but less active than gentamicin or dibekacin. However, fortimicin A is active in vitro and in vivo against several strains of bacteria resistant to other aminoglycoside antibiotics.[80] The systematic modification of aminoglycoside antibiotics continues as a means of eliminating the enzymatic deactivation of these agents by resistant organisms. A number of 2-Deoxy-fortimicin A and B derivatives were synthesized by chemical means. 2-Deoxy-fortimicin A is 35 percent more active than fortimicin A, while retaining the same antibacterial spectrum.[81] Against gram-negative clinical isolates, fortimicin A is similar to amikacin.[82] In clinical studies, fortimicin A showed good efficacy against gentamicin and amikacin resistant gram-negative organisms with no severe side effects.[83] Selective deacylation of fortimicin B (des-glycyl-15) resulted in a series of derivatives, the best of which (4-N-sarcosyl-and 4-N-B-alanyl-fortimicin B) were about 50 percent as active as fortimicin A with no increase in antibacterial spectrum.[84] O-Demethyl-fortimicin A (A49759), when tested in vitro, was two-to-four fold more potent than fortimicin A against most species, with the exception of P. aeruginosa.



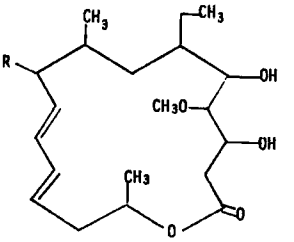
15

It also was resistant to most inactivating enzymes.[85] A series of chlorinated fortimicin A derivatives was reported, as well as their dehalogenation products. Of these 2-deoxy-fortimicin A had the best activity. This study showed that the C-5 oxygen function was required for optimum activity, while the displacement of the C-2 OH with a chloro group did not reduce activity.[86] A series of demethyl derivatives of istamycin A, a new member of the fortimicin group, was prepared by an extension of the total synthesis of this antibiotic. The analogs still bearing the 4-N-methyl group had interesting activity, indicating that this structural feature is essential for activity.[87] Similarly, 3-O-methyl-istamycin B and 2"-N-formimidoyl-istamycin B derivatives were prepared from istamycin B. All derivatives displayed good activity with the 3-O-methyl analogs being more active than istamycin B against P. aeruginosa.[88] SF-1854, an N-formyl-fortimicin-A, was less active than fortimicin A.[89] Sannamycin C and its 4-N-glycyl derivative were isolated from Streptomyces sannanensis, which also produces sannamycins A and B. The 4-N-glycyl derivative has strong and broad activity against aminoglycoside resistant bacteria.[90] The disaccharide antibiotics X-14847 and 3-trehalosamine were recently isolated and demonstrated weak activity.[91,92] The pseudodisaccharide neamine was modified in the 3' and 4' positions to give aminodeoxy neamine analogs, which were less active than neamine against both gram-positive and gram-negative organisms.[93] The 4'-deoxy analogs of neomycin and paromamine were synthesized and found active against resistant E. coli and P. aeruginosa.[94] The 2',3'- epimino analogs of neamine, ribostamycin and KM-B were inactive against non-resistant strains.[95] Several 6"-deoxy and 4", 6"-dideoxy-dibekacin and amikacin analogs, as well as 3',4',4",6"-tetradeoxy and 3',4',6"-trideoxy-amikacin, displayed activity similar to their respective parent compounds. Deoxygenation of the 4" or 6" position reduced antipseudomonal activity.[96] Antibiotic SCH 21420, an aminoglycoside derived from gentamicin-B, is similar to gentamicin-B and amikacin in its antibacterial activity.[97]

Macrolides - The mycinamicins (I, II, III, and IV, 16) were isolated from Micromonospora griseorubida and shown to be a new macrolide family of antibiotics.[98,99] New 3- and 4"-acyl derivatives of tylosin were obtained by microbial transformation by Streptomyces thermotolerans. The antibacterial and antimycoplasmal activities of these acyl derivatives were enhanced by the 4"-acyl group.



16: I = R=H, R'=
II = R=OH, R'=
III = R=H, R'=C=C
IV = R=OH, R'=C=C

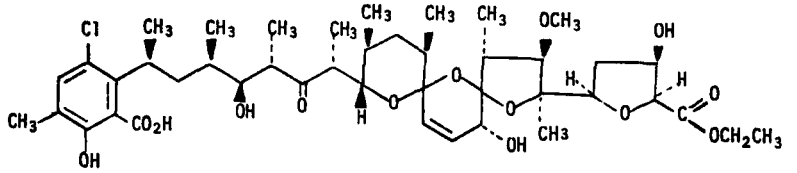


17: P1-I R=O
P1-II R=

The 3-acetyl-4"-isovaleryl derivative showed enhanced oral activity and was effective against S. aureus infections in mice.[100,101] Bioconversion of platenolide glycosides using S. hygroscopicus resulted in the isolation of derivatives acylated at the 3-position, as well as derivatives hydroxylated at C-14.[102] Three novel glycosides of platenolides (P1) I and II (17) were obtained by bioconversion of turimycin by S. hygroscopicus.[103] A series of 4"-O-acyl derivatives of the biotransformation product 4"-O-deacyldeltamycin was produced by chemical means to give a series of substituted phenacetyl derivatives which showed good antibacterial and antimycoplasma activity.[104] A sizeable series of chemically produced acyl derivatives of leucomycin A5 (LM A5) was evaluated, and the 3"-propionyl LM A5 (MS-19-Q) had the best activity against gram-positive macrolide resistant strains, mycoplasma and anaerobes when compared to josamycin and LM A5.[105] This enhancement of activity was ascribed to an improved uptake by the bacterial cell.[106]

A new and simple method of "hybrid" biosynthesis of macrolides was described as an alternative to mutasynthesis and as a means of biosynthetic design of novel antibiotics. This method requires inhibition of a specific biosynthetic enzyme and bioconversion of the accumulated intermediate by a second organism.[107] The structure of macrolide 23672 RP was reported from x-ray studies and shown to be a 14-membered lactone resembling lankamycin.[108] The total synthesis of macrolide antibiotics is of continuing interest for the synthetic chemist. Two groups reported the synthesis of macrocyclic lactone A26771B.[109,110] The stereospecific total synthesis of carbomycin B and josamycin was reported and accomplished starting from D-glucose.[111]

Ionophores - The ionophoric antibiotics of the polyether group continue to be the subject of structural studies. The chemical structures of antibiotic No. 6016,[112] lonomycins B and C,[113] leuseramycin,[114] A28595B [115] and X-14766A [116] have been reported. Antibiotic X-14766A (18), a polyether produced by Streptomyces malachitofuscus, is the first chlorinated member of this class. The ionophores continue to present a challenge to the synthetic chemist. A series of papers describes a new and highly convergent synthesis of monensin.[117] Another synthesis of lasalocid (X537A) has also been reported.[118]



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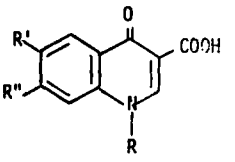
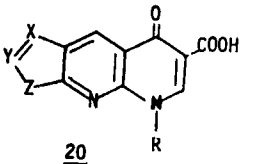
Glycopeptides - The structure of the glycopeptide ristocetin was reported in detail using NMR and chemical data.[119-121] The mode of action of ristocetin as an inhibitor of cell wall synthesis by interacting with key cell wall constituents was studied using NMR to show complexation of the cell wall peptide analog Ac-D-ala-D-ala. The binding studies showed interactions similar to those observed previously with vancomycin.[122] The structures of glycopeptides avoparcin -α and -β were disclosed.[123] The details of a preliminary

chemical characterization of A35512B, which was isolated from a family of glycopeptides produced by Streptomyces candidus, were described. The antibacterial activity was typically high against gram-positive bacteria. The chemical constituents indicate that this antibiotic is related to the ristocetin family.[124,125]

Synthetic Agents - The antibacterial activity of miloxacin showed good in vitro and oral in vivo activity against a variety of gram-negative bacteria, including members of the Enterobacteriaceae and Haemophilus. Miloxacin (19a) had activity comparable to oxolinic acid (19c) and was considerably more active than nalidixic acid (19b) and significantly better absorbed and excreted than oxolinic acid.[126,127]



19: a: R=CH_3O- R', R"=-O-CH_2-O-
b: R=C_2H_5- R', R"=-O-CH_2-O-
c: R=C_2H_5 R'=H, R"=CH_3



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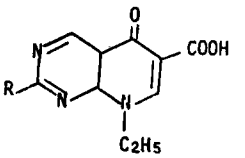

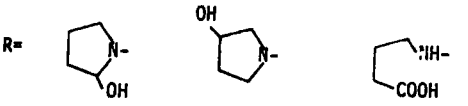

21

Several new naphthyridine derivatives were synthesized as potential antimicrobial agents. The imidazo, triazolo and other heterocyclic condensed 1,8-naphthyridine derivatives (20) showed inhibition of gram-positive and gram-negative bacteria in vitro similar to pipemilic acid, but were less active against P. aeruginosa.[128,129] A study of the metabolite of piromidic acid showed that some of the derivatives bearing hydroxylated basic side chains (21) had increased gram-negative antibacterial activity.[130] AM 715, a nalidixic acid analog, was reported to have good in vitro activity against gram-positive and gram-negative organisms and was superior to nalidixic acid, pipemidic acid and miloxacin in the treatment of experimental mouse infections. Pharmacokinetic experiments showed AM 715 to have good oral absorption and urinary excretion (90 percent) in human subjects. Am 715 was found to be clinically useful against urinary tract infections.[131-133] The antibacterial activity of phosphanilic acid, an analogue of sulfanilic acid, was studied alone and in combination with other synthetic antimicrobial agents. Phosphanilic acid showed antipseudomonal activity against a wide variety of organisms, including P. aeruginosa in mice.[134]

Miscellaneous Antibiotics - Other compounds recently reported to have antibacterial activity are listed in Table 1. The structure for setomimycin, a previously reported antibiotic,[144] was published.[145] Griseorubins are a new family of antibiotics having in vitro activity against gram-positive and gram-negative bacteria,

mycobacteria, mycoplasma and protozoa. They also have activity against certain tumors in rodents.[146] Manoalide 1 is a new sesquiterpenoid antibiotic isolated from the marine sponge, Luffariella variabilis.[147]

TABLE 1. NEW ANTIBIOTICS

Antibiotic	Producing Organism	Activity*	Reference
A21978C	Streptomyces roseosporus	G+	135,136
FR-900130	Streptomyces catenulae	G+	137
Bu-2349A	Bacillus cereus	G+,G-	138
Bu-2545	Streptomyces sp.	G+, AA	139
Dnacins, A,B	Actinomycete	G+, G-, AF	140,141
Nodusmicin	Saccharopolyspora hirsuta	G+, G-, AA	142
Octapeptin D	Bacillus sp.	G+, G-	143

*G+ (gram-positive); G(gram-negative); AA (anaerobic); AF (acid fast)

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Chapter 13. Human Antiparasitic Agents

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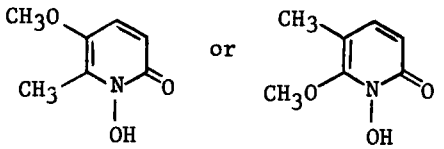
Publication of the 4th Edition of "Burger's Medicinal Chemistry" in 1979 provided excellent, in depth, reviews of the chemotherapy of protozoal and helminth diseases,[1] while a succinct summary of the physician's needs for treatment of tropical diseases was given by Gilles.[2] Many summaries of the occurrence and therapy of parasitic diseases appeared during the year, often prompted by the physician's need for information on the problems created by increasing holiday travel[3] or by urban populations of immigrant origins.[4] Proceedings of the Third Janssen International Symposium entitled "The Host-Invader Interplay" have been published, and the section headed "Drugs and Vaccines" is specially recommended.[5] A review paper discussing The Molecular Basis for the Action of Some DNA-Binding Drugs contains sections covering quinacrine, ethidium and hycanthone/lucanthone.[6]

Protozoal Diseases

Two reviews have covered the side effects[7] and genetic toxicity[8] of anti-protozoal agents, and book chapters on anti-protozoal compounds (hydroxystilbamidine and quinine) have been published.[9]

Trichomoniasis - Nitroimidazoles continue to be the main clinical treatment for trichomoniasis, and single-dose therapy of 1.8 - 2.0 g with either metronidazole[10] or tinidazole[11] is now preferred to an extended 5 to 7-day treatment with lower doses. The general mechanisms of selective toxicity by nitroimidazoles have been reviewed,[12] with special attention being given to the finding that DNA binding by reduced metronidazole metabolites is specific for a high A+T content, as found in Trichomonas and Entamoeba.[13] Biochemical techniques have shown that changes in the hydrogenosome structure and metabolism accompany the aquisition of metronidazole resistance in T.foetus.[5] This differs from resistant forms of T.vaginalis, which show other changes only revealed in the presence of oxygen.[14]



(1)

Three antibiotics show activity against T.vaginalis, either in vitro (toromycin,[15] and a cyclic hydroxamic acid (1)[16]) or when used clinically as vaginal tablets (pentamycin).[17]

Giardiasis.[18] A recent review indicates that only three drugs are recommended for giardiasis. These are quinacrine, metronidazole and furazolidone, with furazolidone being particularly suitable for young children.[19] Metronidazole and its analogs are effective, even with short-term therapy[20] but remain unapproved in the USA for use in Giardia infections. A short-term, in vitro test using the "minimal immobilizing concentration" as the parameter for drug effects has shown that nitroimidazoles act directly on Giardia at concentrations of 0.1 µg/ml.[21] The availability of an axenic culture system has enabled detailed studies on the respiratory metabolism of G.lamblia to be carried out.[22]

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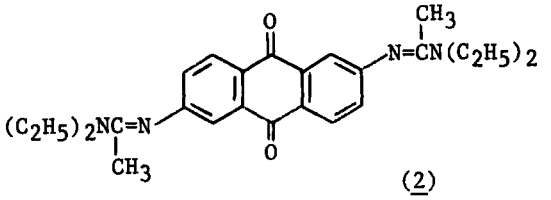
Amoebiasis - A thorough review of all anti-amoebic agents has appeared.[1] As with trichomoniasis and giardiasis, short-term therapy (3 days at 1.5g/day) with nitroimidazoles (ornidazole) has been shown to give almost complete cure of human intestinal amoebiasis.[23]

Many bis-amidines of 2,6-diaminoanthraquinones show good anti-amoebic (and anti-trichomonal) activity in experimental models, and the same lead compound (2), with activity as good, or better than, metronidazole against E.histolytica, has been described independently by two groups.[24,25] The structure/activity relationships of this series are fully described.[24]



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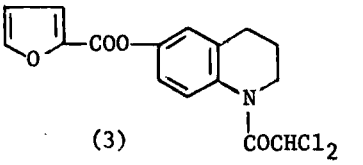
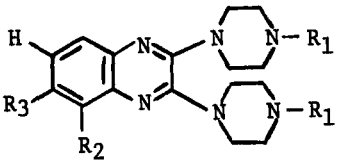
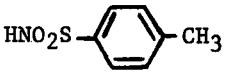


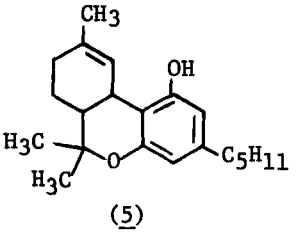
(3)

Preliminary clinical trials and experimental results have shown that the tetrahydroquinolyl ester quinfamide (3) has high anti-amoebic activity when given as a single oral dose.[26] Several series have been described which show good anti-amoebic activity in laboratory models. Polymethoxy-2-nitrobenzofurans[27] and the amidines and sulphonamides of 5-and 6-amino-2,3-bis (4-alkyl-1-piperazinyl) quinoxalines (4)[28] each show active compounds in the in vitro models, but the latter series is too toxic for use in man. Some newer furan haloacetamides with in vitro anti-amoebic activity have been synthesized.[29]



	R_1	R_2	R_3
(4) (a)	CH_3	H	$N{=}CHN(CH_3)_2$
(b)	CH_3	$N{=}CHN(CH_3)_2$	H
(c)	$CH_2CH_2CH_3$	H	$N{=}CHN(CH_3)_2$
(d)	CH_3	H	

Amphotericin B has most often been used clinically in treatment of primary amoebic meningoencephalitis caused by Naegleria fowleri.[30] It is usually ineffective when used alone and both clinically and experimentally is used in combination with other agents such as minocycline, tetracycline[31,32] and rifamycin.[33] In vitro and in vivo models indicate that many derivatives of Δ^9-tetrahydrocannabinol (5) will act against N.fowleri and have the advantage that they accumulate in the central nervous system.[34] Trimethoprim will inhibit non-pathogenic strains of N.fowleri at 4 µg/ml, but virulent strains are resistant to levels of 400 µg/ml. This constitutes an in vitro test to distinguish between virulent and non-virulent strains and indicates that pathogenicity may be linked with folate metabolism.[35]



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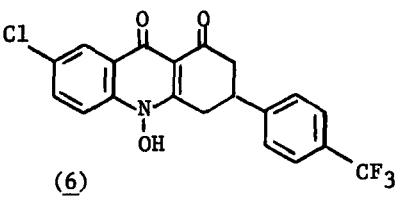


(6)

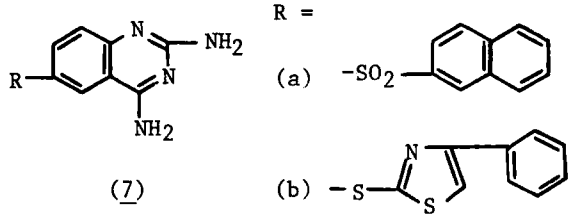
Malaria.- Two reviews of antimalarial chemotherapy have been published recently,[1,36] together with a comprehensive review of the biochemistry of all Plasmodium species.[37]

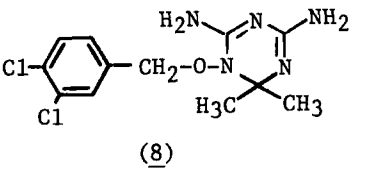
The development of mefloquine continues, with studies on its pharmacokinetics in dog and man,[38] and a report noting its lack of mutagenicity in a bacterial fluctuation test.[39] In a bacterial system, mefloquine binds to the cell membrane, and affects its function and integrity.[40]

The structure/activity relationships of 10-hydroxy-3,4-dihydroacridine-1,9(2H, 10H) diones as anti-malarials[41] (and anti-coccidials)[42] and the development of floxacrine (6) from the series are described. The antimalarial properties of the 2-hydroxy-3-alkyl-1,4-naphthoquinones are reviewed,[43] and a pattern recognition method applied to this series enables active and inactive compounds to be distinguished by using their partition coefficients and molecular orbital energies.[44]

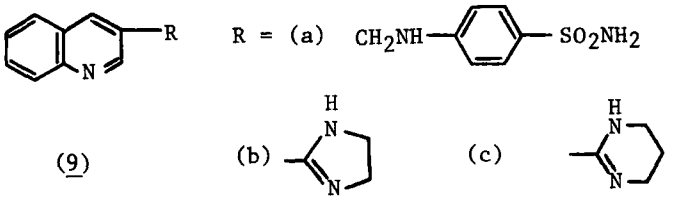
Evidence suggests that ferriprotoporphyrin IX, generated by malarial metabolism,[45,46] fulfills the criteria demanded of a chloroquine receptor, and the complex so formed could affect parasite membranes by its lytic properties.[47]

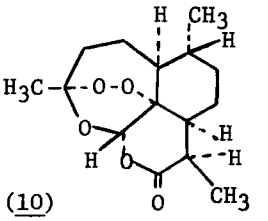
Additional chemistry on the 2,4-diamino-quinazoline series has given rise to folate inhibitors, which are entering the preclinical phase of testing as antimalarials.[48] The preferred compound is 2,4-diamino-6-(2-naphthylsulfonyl)-quinazoline (7a), which is active against both P.falciparum and P.vivax and several strains of drug-resistant malaria. Its activity is potentiated by sulphonamides. Another compound of this type which has shown activity against P.berghei is 2,4-diamino-6-((4-phenyl-2-thiazolyl)thio)quinazoline (7b).[49] The development and eventual discarding of the antifolate compound clociguanil (8) has been documented.[50]





Various 3-substituted quinolines (9) have shown activity in the P.berghei test system.[51,52] Novel oxadiazoles and thiadiazoles have been patented as antimalarial (and anti-coccidial) agents.[53] A report, in English, describes the antimalarial properties of Qinghaosu (10), an ancient Chinese remedy which shows activity against P.vivax and P.falciparum when given orally or intramuscularly.[54]



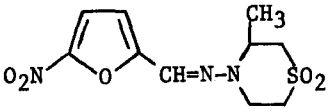


Two review papers examine drug resistance problems[55] and its genetic mechanisms.[56] Further work is presented on the use of primaquine in the form of liposomes or peptide derivatives.[5] Several authors describe excellent in vivo[57] and in vitro test systems for use in drug testing.[58,59] The P.falciparum in vitro system has been used to test novel inhibitors which may give leads to future chemotherapy.[60,61]

Trypanosomiasis - Reviews have appeared which cover the chemotherapy of all trypanosome species,[1] or of T.cruzi only.[5,62] A unique paper has appeared which illustrates the effects of trypanocides on the fine structure of the target organisms.[63]

In vitro assays for drug activity have been described, using radioactive labelling, and used to evaluate a series of 2-acetylpyridine thiosemicarbazones against T.rhodesiense.[64,65]

Many experimental studies with T.cruzi are being carried out with nifurtimox (11), which has been shown to be mutagenic in bacteria.[66] The production of the nitrofuran radical, then superoxide anion and peroxide, are thought to be important in its killing action.[67] The activity of (11) extends to other trypanosomes and Leishmania species.[68] The related nitrofuran SQ 18506 (12) has been shown to inhibit RNA, DNA and protein synthesis in T.cruzi.[69]



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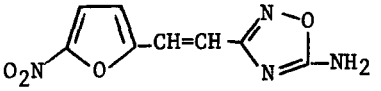
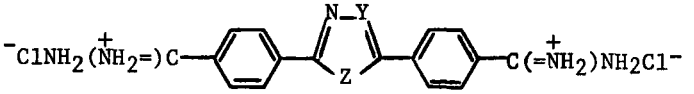


(12)

Allopurinol is effective against T.cruzi in both in vitro and mouse in vivo systems[70] and is converted to its nucleotide by parasite enzymes.[71] T.brucei and T.rhodesiense also carry out this conversion and are inhibited by allopurinol under in vitro conditions.[72] Oxazoles (a) and thiadiazoles (b) (13) have been synthesized which give cures of T.rhodesiense in mice at 3 mg/kg.[73]

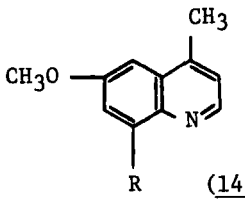


(13)

	Y	Z
(a)	CH	O
(b)	N	S

Leishmaniasis - New experimental models for visceral and cutaneous leishmaniasis in rodents have been developed and validated with an extensive range of standard inhibitors and experimental compounds.[74,75] The use of liposomes for the targeting of anti-leishmanial compounds continues.[5,76]

Recent interest in anti-leishmanial agents lies in the lepidines (4-methyl-6-methoxy-8-aminoquinolines (14)).[77] Many other analogs of this highly active series are being made and tested.[78-80]



R = (a) $-NH(CH_2)_6NHCH(CH_3)CH_2CH_3$

(b) $-NH(CH_2)_6NHCH_2CH(CH_3)_2$

(14)

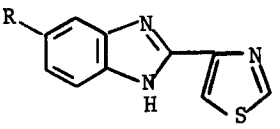
Pneumocystis - Trimethoprim (TMP)/sulfamethoxazole (SMX), given orally or parenterally, is the drug combination of choice for treatment or prophylaxis of Pneumocystis carinii pneumonia, especially in immuno-supressed patients. Pyrimethamine/sulfadoxine is less effective for therapy but ideal for prophylaxis.[81] In a rat model, 6 weeks of TMP/SMX therapy (50 mg/kg/day) does not eradicate the infection but supresses it.[82] An in vitro maintenance system, useful for biochemical and chemotherapeutic studies, using P.carinii from rats, has been used to show that TMP and SMX are not immediately lethal to the organism as measured by a dye-uptake test.[83,84]

Helminth Diseases

General - A major review by Islip gives almost complete chemical coverage of anthelmintic chemotherapy.[1] Biochemical mechanisms for the action of current anthelmintics have been discussed, and special attention was drawn to the neuromuscular system, energy production, electron transport and tubulin polymerisation processes as metabolic areas where novel inhibitors may still be found.[85,86] Recently observed side-effects of anthelmintic drugs are listed.[87] A few more papers have appeared reporting studies of the drug-metabolizing enzymes of nematodes, cestodes and trematodes.[88,89]

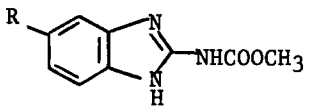
The present excitement in anthelmintic chemotherapy comes from the development of wide-spectrum compounds, and some of these will be discussed separately before describing the species-specific inhibitors.

Benzimidazoles - Following the activity of the thiazolyl-benzimidazoles (thiabendazole (15a) and cambendazole (15b)) against gastrointestinal nematodes, the introduction of the newer benzimidazole carbamates (16 a-c) extended this activity into filarial nematodes, cestodes and trematodes. The benzimidazoles can now be considered as highly successful wide-spectrum anthelmintics, and more antiparasitic patents appear for this series than for any other.



(15) R = (a) H — thiabendazole

(b) $NH-C(=O)-O-CH(CH_3)_2$ — cambendazole



(16) R = (a) $-C(=O)-C_6H_5$ — mebendazole

(b) $-C(=O)-C_6H_4-F$ — flubendazole

(c) $-S-C_6H_5$ — oxfendazole

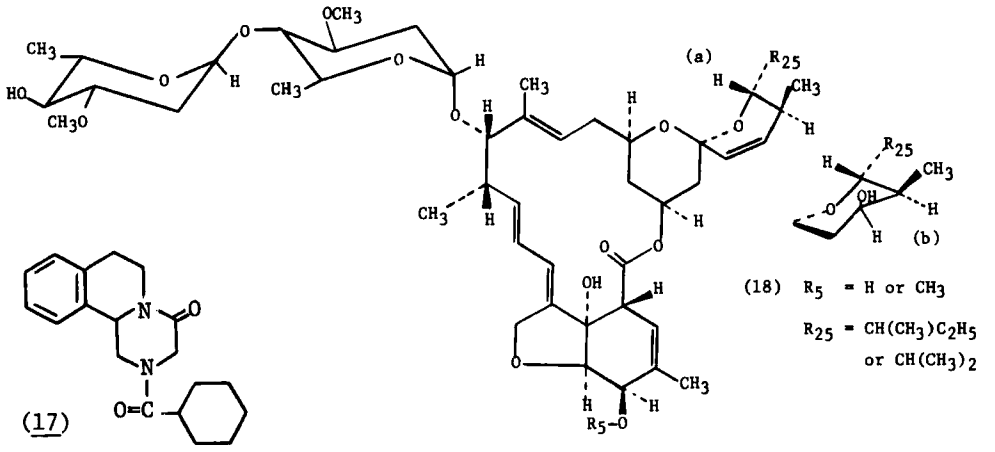
Clinical studies have confirmed the activity of (15a) and (16a) against trichuriasis, hookworm, strongyloidiasis and ascariasis.[90,91] Although several benzimidazoles are effective against larva migrans induced by Toxocara infection, thiabendazole can no longer be recommended.[92,93] When (16 a,b,c) are tested against Trichinella spiralis in the mouse, it becomes increasingly difficult to sterilize the infection as it progresses through the four stages of its life cycle.[94-96] The efficacy of benzimidazoles as macrofilaricides by parenteral routes is confirmed, but orally they are usually ineffective.[97,98] Although (16a) is the only chemotherapeutic agent which can be recommended for therapy or pre-operative sterilization of the cysts of Echinococcus, it is not completely satisfactory.[99,100] Flubendazole (16b) is also effective against trematode infections in man.[101]

The binding of benzimidazoles to tubulin has been suggested as a mechanism of inhibition. Tubulin has now been isolated from cestode[102] and nematode material, and work with Ascaris tubulin provides a theoretical basis for differential binding and inhibition.[5,103]

Levamisole - Although there is no doubt about the direct anthelmintic activity of levamisole, the majority of work now published on this compound relates to its immunomodulatory properties.[104] Its success as an antiparasitic agent may involve its immunostimulant properties in an animal immunodepressed by infection.[105]

Praziquantel - First known as a veterinary anti-cestode agent, praziquantel (17) is emerging as an exciting anti-trematode agent with a special place in the possible mass therapy of all species of schistosome found in man.[106] A 99.5% cure rate against human cases of S.japonicum has been reported by a Chinese group.[107] Praziquantel has been successfully used for many human cestode infections,[108] and Japanese workers have found it to be active against experimental Clonorchis infections,[109] although it has no effects on Fasciola.

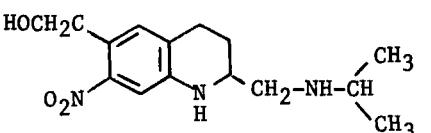
Electron microscopy of cestodes[110] and trematodes[111] shows that (17) induces vacuolization and "blebbing" in specific areas of the tegument and leads to complete disruption of the surface layers.[112] Gross changes in the metabolic patterns accompany the morphological damage.[111] Praziquantel is taken up rapidly, and impaired movement is followed by paralysis.[113] Known anti-cholinergic agents cannot prevent the contractions induced by (17).[113] In schistosomes, paralysis would seem to follow an influx of Ca^{2+} to the parasite's musculature.



Avermectins - The anthelmintic activity of avermectins (18) extends to at least eight families of nematodes, including the Filariidae, and this antibiotic series has recently been extended to include the semi-synthetic 22,23-dihydro derivative (18b) ivermectin.[114,115] At least five patents in 1980 covered Merck avermectin derivatives. Anthelminthic activity is shown at doses well below 1 mg/kg, both orally and parenterally. Avermectin B_{1a} immobilizes both parasitic and free living nematodes.[116] In vivo work with Trichinella spiralis has shown that the effects of avermectins cannot be supressed by agents known to block cholinergic nerve transmission, indicating that an alternative neurotransmitter system is affected.[117] It is proposed that in Ascaris, avermectin B_{1a} simulates inhibitory neuronal input, either by acting as a GABA agonist or by stimulating GABA release from presynaptic inhibitory terminals.[118]

Schistosomiasis - A summary of the treatment of schistosomiasis for the physician has appeared,[118] together with a short review of the mode of action of some anti-schistosomal drugs.[119]

Although it seems that praziquantel could revolutionize the mass therapy of schistosomiasis, there are several other compounds which are also of great interest. Clinical papers describe the successful treatment of S.mansoni using a single dose, or one-day oral treatment with oxamniquine (19), but S.haematobium is not affected.[120,121] S.haematobium in young children is best treated with niridazole, but lower doses (12.5 mg/kg/day) over an extended period are required to avoid adverse neurological reactions.[122] Sustained release of niridazole from silicone rubber implants may therefore prove worthy of clinical trial.[123]



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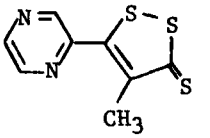


(20)

Extended clinical trials have shown that oltipraz (20) is as effective as (19) and niridazole against S.mansoni, S.haematobium and S.intercalatum, and a dose as low as 2g given during a single day is effective.[124,125]

Preliminary testing against S.mansoni in mice has shown activity in 3,5-dioxo-2,3,4,5-tetrahydro-6-(5'-nitro-2'-thiazolylamino)-1,2,4-triazine,[126] N4-(N-arylglycyl)-sulfanilamides acetylated or trifluoroacetylated at the glycyl nitrogen,[127] and 3-mercaptotriazoles substituted with the 8-hydroxyquinoline moiety in the 5-position.[128] 2-Chloro-4-(4-alkyl-1-piperazinyl) benzenemethanol N-oxides have been patented as antischistosomal agents.[129]

Evidence is accumulating that many anti-schistosomal compounds act on neurotransmission or neuromuscular systems of the parasite.[86] With the exception of (17) and the benzodiazepine Ro11-3128[130] which interact with 5-hydroxytryptamine uptake inhibitors in S.mansoni,[131] all such compounds interfere with a cholinergic system. Interaction with this latter system has already been shown for hycanthone, oxamniquine and metrifonate.[86] Hillman and colleagues have made detailed studies of dansylated acetylcholine analogs, acetylcholine mustards and phosphonium compounds as anti-cholinergic compounds in S.mansoni.[132,133]

Other Trematode Infections - Metabolic studies of the effects of closantel on Fasciola[134] and of bithionol and menichlopholan on Clonorchis, Metagonimus and Paragonimus[135] have appeared, together with a clinical study of the successful treatment of Fasciolopsis with niclosamide and dichlorophen.[136]

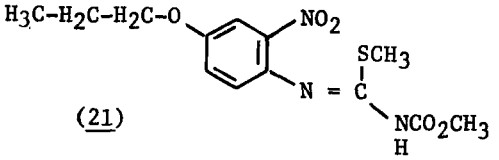
Cestodes - Mesocestoides infection in man has been successfully treated with paromomycin.[137] Substituted 3,5-dibromosalicylanilides have been synthesized and their structure-activity relationships against Hymenolepis diminuta examined.[138] Activity, better than niclosamide, was found in some members of this series with single oral dose levels of 10 mg/kg.

Filariasis - The technique for determination of microfilarial density in human skin snips during the treatment of onchocerciasis has been standardized.[139] Onchocerca gibsoni and O.gutterosa in cattle provide good therapeutic models for O.volvulus with most of the standard drugs tested.[140] The use of a low dose of diethylcarbamazine (DEC), as a provocative day test for nocturnally periodic microfilariae is recommended.[141] An in vitro assay technique using O.volvulus microfilariae, from skin snips, has shown that the action of DEC is that of a neuromuscular blocking agent.[142]

DEC remains the most effective microfilaricide given either orally for lymphatic filariasis[143] or topically for onchocerciasis.[144] Metrifonate is also microfilaricidal, and its reduced side effects probably reflect its lower efficacy against microfilaria.[145] The slow macrofilaricidal effect of suramin on O.volvulus was demonstrated by excision of nodules containing adult worms from patients treated six weeks earlier.[146] Several enzymes from adult filariae are inhibited by suramin,[5,147] and folate metabolism has been noted by Jaffe as an area where differential inhibition of filariae may be possible.[5]

Gastrointestinal nematodes - A new primary screening test, capable of detecting broad-spectrum anthelmintic activity, uses the parasitic stages of Nippostrongylus brasiliensis growing in an in vitro system.[148]

By the use of Ascaris[149] and levamisole-resistant mutants of Caenorhabditis elegans,[150] separate authors have concluded that the pharmacological specificity of the nematode cholinergic receptor is most like a vertebrate nicotinic ganglionic receptor. The levamisole mutants show no changes in their response to avermectins, so confirming that the latter do not affect the same receptor as levamisole.



(21)

A pinworm agent (Sch. 23154) (21) active against experimental infections of Syphacia obvelata is described.[151]

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Chapter 14. Antineoplastic Agents

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Introduction - A literature survey on the topic of antineoplastic agents resulted in the citation of approximately 2000 articles or reviews published in 1980, a reduction of approximately 5% from 1979. A listing of agents under clinical investigation in the United States is maintained by The National Cancer Institute (NCI) and is available for distribution,[1] and participation of medicinal chemists in a continuing search for new leads for anticancer drug development is solicited by The Drug Synthesis and Chemistry Branch of NCI.[2]

Reviews were published on anthracyclines,[3] AMSA,[4] DDMP,[5] o,p'-DDD,[6] interferon,[7] retinoids,[8] carminomycin,[9] L-asparaginase,[10] 1-hexylcarbamoyl-5-FU,[11] FD-1,[12] and nitrosoureas.[13]

Articles of general interest have been published on new anticancer drugs,[14] congener synthesis,[15] new concepts in chemotherapy,[16] modeling of drug delivery to brain tumors,[17] the hypoxic tumor cell,[18] NCI's natural products program[19] and new screen,[20] usefulness of murine tumor models for identifying new agents,[21] membranes and cancer therapy,[22] interaction of human serum albumin with anticancer agents,[23] oncostatic-antibody complexes,[24] and protease-activated prodrugs.[25]

Selected Highlights - Among the many publications on various aspects of anticancer drug development during the year, certain reports were particularly notable in the judgement of the author and are emphasized here. A more complete review of the subject appears in subsequent paragraphs. Comparison of structure-activity relationships of nitrosoureas active against L1210 leukemia with their toxicities indicated that neutral agents with octanol/water partition coefficients of -1.5 to -2.5 might have better therapeutic indices than those presently in use.[26] Mitomycin C disappeared rapidly only in cultures of hypoxic EMT6 cells, suggesting that agents capable of bioreductive alkylation may selectively kill the hypoxic cell component of solid tumors and thus be useful adjuncts to current drug regimens that are effective against oxygenated cells.[39] Clinical demonstration that cross-resistance between mustard-type alkylating agents does not exist in some tumors was found when cyclophosphamide-resistant, advanced breast cancer responded to treatment with peptichemio.[49] Direct, chemical evidence that DNA cross-linking is a product of alkylation with busulfan was presented.[59] A "double-barreled" masked precursor hypothesis was supported by observations that xylosyl-5-fluorocytosines substituted at position 3' by a good leaving group exhibited Ara-C type activity against Ara-C sensitive tumor cells but acted as 5-fluoropyrimidines in Ara-C resistant cell lines.[73] Methotrexate polyglutamates, which are readily formed in human tumor cells and bind to dihydrofolate reductase, may be selectively retained in cells and may be important determinants of duration of action and cytotoxicity of methotrexate in human solid tumors.[90] A study of the interaction between adriamycin and phospholipids indicated similar association constants for such complexes and for DNA complexes; the data suggest competitive behavior between a membrane site and target DNA,

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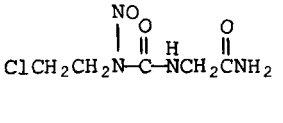
and consequently, lipid components of cell membranes may be an important determinant in the behavior of this agent.[101] Ansamitocins, which are new mitotic inhibitors, demonstrated high activity against experimental tumors in vivo and are possible alternatives to vinca alkaloids in treatment of human tumors resistant to the latter.[109] Vitamin C, which in combination with copper ions exhibited carcinostatic effects against sarcoma 180 tumors in mice, was toxic to malignant melanoma cells in culture; the selective toxicity possibly resulted from the elevated copper concentration in melanoma cells, since no preferential effect of the vitamin against any other type of cancer cells in culture has been demonstrated.[147] Three intercalating 7H-pyrido[4,3-c]carbazole dimers with rigid bis(ethylpiperidyl) chains were highly active against LL; antitumor activity of this class of agents was found to be strongly dependent on position of attachment, nature of the linking chain, and its rigidity.[159] A quantitative structure-activity study of AMSA derivatives led to the conclusion that mutagenic and antitumor activity can be readily separated in the series by appropriate choice of substituent and adjustment of overall lipophilic/-hydrophilic balance.[161] In a limited number of patients with stage III and IV ovarian carcinoma, a dramatic, 70% complete regression rate was obtained with the new agent penberol (7), cis-beta-4-pentoxybenzoyl-beta-bromoacrylic acid.

Alkylating Agents - A quantitative structure-activity relationship for 90 nitrosoureas was formulated,[26] and 1-(2-chloroethyl)-1-nitroso-3-(methylenecarboxamido)urea(1), 2-3-(2-chloroethyl)-3-nitrosoureido-ethylmethanesulfonate, and chlorozotocin were effective against DMBA-induced mammary cancer.[27] Nitrosourea-phensuximide derivatives were synthesized as central nervous system agents,[28] and FCCNU(2) showed high toxicity but little activity against intracerebral(IC) L1210 leukemia(LL).[29] N'-(1-Benzyl-4-piperidinyl)-N-(2-chloroethyl)-N-nitrosourea hydrogen maleate was active against IC LL and an ependymoblastoma,[30] and 1-[2-[(2-chloroethyl)thio]-ethyl]-3-cyclohexyl-1-nitrosourea, an active interstrand DNA cross-linking agent, was active against LL.[31] A 2-chloroethylnitrosourea derivative of N-deacetylmethylthiocolchicine was more active than its parent against LL in vitro.[32] Ethylene glycol was identified from BCNU decomposition,[33] and the higher rates of degradation of some chloroethylnitrosoureas in serum were produced by a non-specific protein-mediated chemical reaction.[34] An interesting report on the reactions of BCNU and CCNU with DNA was published.[35] Therapeutic potentiation of CCNU and ACNU by hyperthermia[36] and 6-thioguanine,[37] respectively, was observed, and the relative mutagenic activities of chloroethyl- and methyl-nitrosoureas in clinical use were determined.[38]



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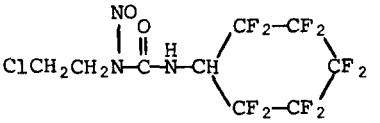

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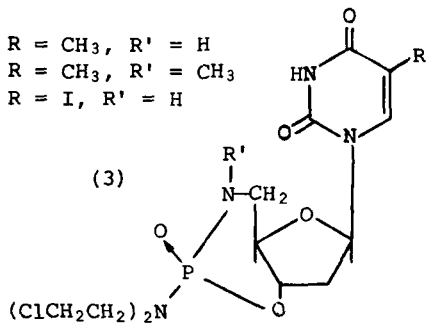
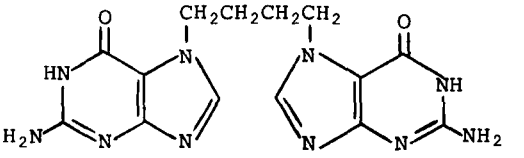
Mitomycin C was found to be selectively toxic to tumor cells made chronically hypoxic, suggesting that such bioreductive alkylating agents have the potential to attack selectively the chemotherapeutically resistant hypoxic cell component of solid tumors,[39] and o-nitrobenzyl and p-nitro

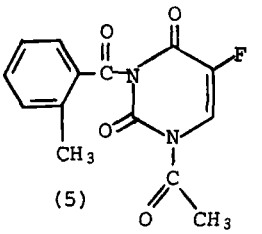
benzyl alcohols, halides and carbamates were prepared as potential bioreductive alkylating agents.[40] The first unambiguous electron spin resonance evidence for the semiquinone metabolite of mitomycin C was reported.[41] Paramagnetic diethyleneimides of urethane phosphoric acids were synthesized and evaluated for antitumor activity,[42] and ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinate(AB-163) was evaluated in a phase I trial.[43]

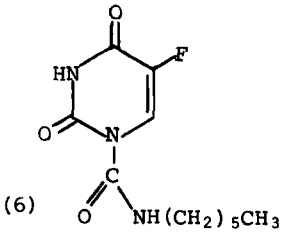
Propanolol mustard, synthesized as a possible lung-specific agent, was active against P388 leukemia(PL) but not against Lewis lung tumor.[44] Several aporphine amide nitrogen mustards,[45] a series of alpha-benzyloxycarbonyl-N,N-bis(2-halogenoethyl)hydrazide derivatives of amino acids,[46] and several 4-p-bis(2-chloroethyl)aminophenylbutyrate esters[47] possessed antitumor activity. A study of the metabolism of chlorambucil was reported,[48] and peptichemio was active against cyclophosphamide(CPA)-resistant, advanced breast cancer, demonstrating that cross-resistance to another mustard-type alkylating agent did not exist in this tumor.[49] Pharmacology studies were reported on 1-(gamma-chloropropyl)-2-chloromethylpiperidine, a new antitumor agent.[50] trans-4-Phenyl-CPA, but not cis-4-phenyl-CPA, was active against LL,[51] and monomeric and polymeric sulfhydryl derivatives of 4-hydroxy-CPA exhibited high and low activity, respectively, against LL.[52] The presence of therapeutically-significant levels of 4-hydroxy-CPA in blood of mice after CPA treatment was demonstrated,[53] and the first report of blood levels of 4-hydroxy-CPA and phosphoramide mustard in humans appeared.[54] Three CPA derivatives of pyrimidine nucleosides(3) were active in vitro,[55] presumably as a result of hydrolysis at C-3' to generate an N-alkylated phosphoramide mustard analog as suggested in last year's chapter for a related derivative. A series of alpha-halo esters of carboxylic acids was synthesized as "soft" alkylating agents, and chloromethyl hexanoate demonstrated anticancer activity.[56] Some iodomethylpyrazole nucleosides were active tumor agents,[57] and three alkylating derivatives of c-AMP stimulated rather than inhibited proliferation of mouse bone marrow stem cells.[58] 1,4-Di(7-guanyl)butane(4) was identified as a product of reaction between DNA and busulfan, demonstrating that busulfan is a cross-linking agent and strengthening the hypothesis that differences in biological properties of alkylating agents result from differences in their reactions with DNA.[59] The antitumor activity of 3-alkyl-1-(4-carboxyphenyl)-3-methyltriazenes rapidly diminished at alkyl chain lengths greater than pentyl,[60] and the adducts of 2,6-dinitro- and 4-cyano-benzaldehyde with p-(3,3-dimethyl-1-triazeno)benzoic acid hydrazide and the corresponding hydrazone of the latter were active against a mouse tumor.[61] 8-Methoxy-15,16-dinor-6,8,10-trichothecatriene 12,13-alpha-epoxide, but not the 12,13-beta-epoxide, was active against PL.[62]





Pyrimidine, Purine and Folate Antagonists - The tumor inhibitory effects of a new derivative of 5-FU, 5'-deoxy-5-fluorouridine,[63] and of a series of 2',5'-dideoxy-and 2',3',5'-trideoxy-nucleosides of 5-FU[64] were reported. Of a number of N-acyl-and N-(alkoxycarbonyl)-5-FU derivatives synthesized, N^1-acetyl-N^3-O-toluyl-5-FU (5) was the most promising and showed higher activity toward various tumors than either 5-FU or Ftorafur (FT-207).[65] 5-Fluoro-2'-deoxyuridine 5'-phosphorodiamidate produced a cytostatic effect comparable to that of FdUrd and FdUrd-5'-phosphate.[66] Tumor inhibition,[67] pharmacokinetics,[68] and metabolism[69] of 1-hexylcarbamoyl-5-FU (6) were reported. Various effects of 5-fluoro-1,3-bis(tetrahydro-2-furanyl)-2,4-pyrimidinedione (FD-1) were compared with those of the corresponding mono-substituted derivative of 5-FU (Ftorafur) after oral administration.[70] Reports on the activation of Ftorafur to 5-FU and gamma-butyrolactone[71] and on the structure of two hydroxylated metabolites of Ftorafur[72] were published. Results of evaluation of cytosine- and 5-fluorocytosine-xylo furanosyl nucleosides with good leaving groups at the 3' position supported the "double-barreled masked precursor" hypothesis,[73] and several conjugates of ara-C with corticosteroids were more active against LL than ara-C.[74] 5-Azidomethyl-, 5-aminomethyl-, and 5-hydroxymethyl-2'-deoxyuridine inhibited LL and sarcoma 180 cells in vitro.[75] Molecular orbital studies of 3-deaza-uridine and -cytidine indicated that their conformational preferences are very similar to those of the parent nucleosides,[76] and 2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl-5-halocytosines were conformationally similar to other ara-C derivatives but quite different from 2'-deoxycytidine.[77] 1-5-Azido- and 1-(5-L-methionyl)amino-2,3,5-trideoxy-beta-D-glycero-pent-2-eno-furanosylcytosine exhibited weak activity against sarcoma 180,[78] and the antitumor activity of a series of 2'- or 3'-amino- or azido-substituted deoxyribonucleosides was reported.[79] Good increases in life spans of mice with mammary carcinoma were obtained with N^6-allyl,-isopropyl, and -propargyl analogs of adenosine,[80] and a new class of tricyclic nucleosides in which the base has a linear geometry was synthesized using pyrrolo[2,3-d]pyrimidine nucleosides as starting materials.[81] Some carbocyclic nucleoside analogs were synthesized and were active against PL in vitro.[82] The toxicity and metabolism of ara-A and 2-fluoro-ara-A were compared in human lymphoblastoid cells,[83] and isocoformycin was shown to block deamination of ara-A and formycin A in vivo.[84]





Several reports were published on plasma levels of 7-hydroxymethotrexate in humans after both low and high doses of methotrexate;[85,86] in one of the studies, 4-amino-4-deoxy-N^{10}-methylpteroic acid was found in both plasma and urine.[86] Evidence was presented for the cytotoxicity of polyglutamates of methotrexate,[89] which were the predominant form bound to dihydrofolate reductase of human breast cancer cells after 24 hours.[90] Synthesis of 11-oxahomofolic acid[91] and 11-thiohomoaminopterin[92] was described, and a convenient synthesis of 10-deazaaminopterin via a pteridine ylide was devised.[93] Various diamino-pyrimidines,[94]-S-triazines,[95]-pyrido[2,3-D]

pyrimidines,[96] and -quinazolines[97] were synthesized and evaluated as inhibitors of dihydrofolate reductase and, in some cases,[96 97] as antitumor agents. Preparation of tetrahydrofolate analogs in high yield and purity was accomplished.[98]

Anthracyclines - Some amino acid and dipeptide derivatives of daunorubicin were more active than the parent against subcutaneously-inoculated LL.[99] Chemical reduction to semiquinones and NADPH-dependent microsomal activation of adriamycin and daunorubicin resulted in covalent binding to DNA,[100] and evidence was presented for a specific complex between adriamycin and negatively-charged phospholipids.[101] Carminomycin-DNA complexes had higher activity against LL than the uncomplexed agent.[102] Structural studies of carminomycins II and III were reported,[103 104] and minor components of the carminomycin complex were identified.[105] N-Acyl derivatives of carminomycin and rubomycin were synthesized,[106] and six anthracyclines were isolated from the bohemic acid complex.[107]

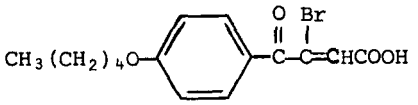
Natural Products and Semi-Synthetics - Ansamitocins, which are maytansinoid antibiotics, were isolated and their antitumor activity determined.[108 109] Macbecins I and II,[110] and streptovaricin U,[111] which also belong to the ansamycin group, were isolated and characterized. Chemical proof for the presence of a free secondary amine in bleomycin (Blm) was presented,[112] and biosynthetic studies indicated that the methyl group of the pyrimidine moiety originates from the methionine-methyl group.[113] The antitumor potency of Blm and some metal complexes was CuBlm>Blm>ZnBlm=FeBlm > CoBlm.[114] A non-protein component of neocarcinostatin was isolated, partially characterized, and shown to be responsible for certain effects on DNA.[115 118] The structure of roridin J, a new macrocyclic trichothecene, was determined,[119] and the mechanisms of action of showdomycin and its reactive moiety, maleimide, were compared *in vitro*.[120] Isolation of tetrocarcins,[121] new antitumor antibiotics, was reported, and prumycin was shown to block cells in G_2 phase.[122] New antitumor antibiotics, neothramycin,[123] saframycin A,[124] and tendomycin,[125] were isolated from *Streptomyces* cultures. BN-183B,[126] a chlorine-containing *Pseudomonas* antibiotic, and the griseorubin complex[127] were both active against LL, and tatumine, a peptide from *Bacillus brevis*, was toxic to sarcoma cells.[128] Sesquiterpene lactones from *Acanthospermum glabratum*[129] and Helenium microcephalum,[130] sesquiterpenoids from *Capsicodendron dinisii*,[131] diterpenes from *Rondeletia panamensis*,[132] and triterpenes and germacranolides[133] from *Elephantopus mollis* were isolated for evaluation as antitumor agents. Antitumor alkaloids were isolated from Korean mistletoe[134] and 21'-oxoleurosine from *Catharanthus roseus*.[135] Structural determinants responsible for the biological activity of emetine, cryptopleurine, and tylocrebrine were investigated.[136] Psorospermin, a xanthone from *Psorospermum febrifugum*, was active against PL,[137] and glycosides of *Asclepiadaceae* were evaluated as antitumor agents.[138 139] Antitumor compounds or extracts were obtained from *Wedelia asperrima*,[140] *Semecarpus anacardium* nuts,[141] *Rubia cordifolia*,[142] and some southern African plants.[143] The major cytotoxic activity of extracts of *Nectandra rigida* was shown to be due to dehydrodiisoeugenol.[144]

The immunostimulant isoprinosine enhanced the antitumor effect of interferon,[145] but its antiviral effect was diminished by certain anticancer agents.[146] Vitamin C was preferentially toxic to malignant melanoma cells, possibly because of elevated copper concentrations in melanoma,[147] and the

polysaccharide levan exerted a direct antitumor effect on Lewis lung carcinoma *in vitro*.[148] 5-*S*-Cysteinyl-DOPA, a melanin precursor, was selectively toxic to tumor cells *in vitro* and *in vivo*.[149]

Of a series of asparagine analogs, the *N*,*N*-dibenzyl derivative showed significant anticancer activity.[150] Several olivomycin A analogs were equal or superior to the parent in potency and efficacy against PL,[151] and analogs of the antitumor lignan steganacin were tested for inhibition of microtubule assembly.[152] Some semi-synthetic, as well as some totally-synthetic, analogs of camptothecin were active against PL, and L-camptothecin was shown to be inactive.[153] Chemical modification of coriolin B[154] and of the ester group of diketocoriolin B[155] gave tumor active derivatives. Derivatives of macrocyclic trichothecenes, verrucarin A and B and roridin A, were active against PL,[156] as was a maltoside analog of chartreusin.[157]

Miscellaneous Synthetic Agents - One member of a series of halogenated analogs of L-fucose and D-galactose was active against LL *in vitro*.[158] 7*H*-Pyridocarbazoles and dimers thereof were synthesized as DNA intercalating compounds, and three of the dimers were highly active against LL.[159] Dipyrido[4,3-B][3,4-F]indoles were also synthesized and found to be active against LL.[160] Quantitative structure-activity relationships were developed for the mutagenic and antitumor activity of AMSA derivatives,[161] and a report on the mechanism of action of AMSA appeared.[162] Phenyl-substituted benzenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide were synthesized independently by two groups and evaluated for anticancer activity.[163 164] 2-Methyl-3-acetoxy-4-pyridone is the most active compound discovered thus far in the pyridone series,[165] and a series of bis(*N*-alkylcarbamate) derivatives of pyrroles[166] and pyrrolizines[167] was prepared and evaluated against PL. 1,4-Benzoquinone derivatives[168] and imides of 3-nitro-1,8-napthhthalic acid[169] were evaluated as antitumor agents, and hydroquinone dialdehydes were active against several ascites tumors.[170] Four steroidal alpha-methylene delta lactones were active *in vitro*,[171] and derivatives of hexestrol and diethylstilbestrol containing various functions were synthesized.[172] A series of ketals, hemithioketals, and dithioketals of nuclear-substituted styryl ketones was prepared as latentiated forms of the ketones.[173] A new agent, penberol(7), was effective against ovarian carcinoma,[174] and thiazolidine-4-carboxylic acid(norgamem) gave responses in several human cancers.[175] Activity against mouse tumors was shown by diazomethyl and chloromethyl ketone analogs of *N*-tosyl amino acids,[176] and L-canavanine was selectively toxic to proliferating cells.[177] *N*-Hydroxyadamantanine was active against PL *in vitro*.[178] Chlorohydroxyacetone benzoate and nitrobenzoates cured Ehrlich tumor in immunocompetent mice,[179] and the mode of action of 1,1'-(methylethanediylidene)dinitrilo-bis(3-aminoguanidine)(8) was fully comparable to that of methyl-GAG.[180] A new group of polymers with antitumor activity was prepared.[181]



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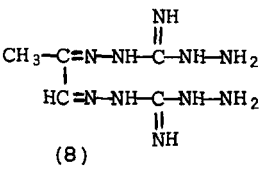

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N-Hydroxymethylpentamethylmelamine,[182] a metabolite of hexamethylmelamine, was active against ovarian cancer cells.[183] HPLC was used in pharmacology studies on DON[184] and 1,4-bis(2-(2-hydroxyethyl)aminoethylamino)-9,10-anthracenedione,[185] and a phase I study on the dihydroxy derivative of the latter was reported.[186] Phosphorylated aldehydes potentiated the activity of CPA against LL, presumably by inhibiting deactivation of aldophosphamide by aldehyde oxidases or dehydrogenases,[187] and maleic anhydride-divinyl ether copolymer anions were effective adjuvants with irradiated LL tumor cell vaccine.[188] Structural similarities and differences in nafoxidine, clomiphene, and tamoxifen, the 3 antiestrogens with clinical efficacy against mammary carcinoma, were reported.[189]

Miscellaneous - The primary structure of E. coli L-asparaginase[190] was determined, and a new antitumor enzyme, L-lysine alpha-oxidase, was purified.[191] WR-2721 inhibited the nephrotoxicity of cis-Pt without altering its antitumor properties,[192] and cis-malonato-diammino platinum (II) inhibited PL in vivo.[193] Titanocene, but not zirconocene or hafnocene, was active against Ehrlich tumor in mice.[194] Ruthenium red[195] and rhodium (II) butyrate[196] inhibited Lewis lung tumor in vivo and HeLa cells in vitro, respectively. The activity of liposome-entrapped actinomycin D,[197] methotrexate,[198] and neocarcinostatin[199] against murine tumors was assessed, and several anticancer agents were microencapsulated in various synthetic matrices.[200-202] A mitomycin C-dextran conjugate demonstrated increased antitumor activity in comparison with the free drug,[203] and cellular uptake of methotrexate-poly(L-lysine) conjugates far exceeded uptake of free drug in drug sensitive and resistant lines.[204]

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Chapter 15. Antiviral Agents

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The last review[1] in this series on antiviral agents concentrated on drugs that affect DNA viruses and, to a lesser extent, on drugs with broad spectrum antiviral activity. It is the purpose of this year's review to cover agents with activity against RNA viruses. During the past year reviews by Sidwell and Witkowski,[2] Shannon and Schabel,[3] Hirsch and Swartz,[4] Hahn,[5] and Smith et al.[6] have appeared which provide broad coverage of drugs active against both RNA and DNA viruses. Additional in depth reviews of specific agents also have appeared and are cited in the text which follows.

The primary problem confronting those who would develop safe and efficacious drugs to treat virus diseases is the necessity to selectively inhibit the apparently few biochemical functions that are uniquely utilized by viruses. Because the replication of viruses requires the use of so many host cellular functions, severe drug-induced toxicity will occur in the host if viral functions are not blocked selectively. Some functions that are uniquely required by viruses which have yielded to selective inhibition include cell surface receptors and virus-coded enzymes.[1] Chemotherapeutic exploitation of such functions definitely is involved in the success of the limited number of drugs currently available and should lead to the development of new, highly effective antiviral drugs.

NEW RNA VIRUS-INHIBITORY COMPOUNDS

A review of literature published up to 1978 on substances active against RNA viruses has appeared.[2] This portion of the current review will consider the more recent compounds which have been reported to have a significant degree of activity against RNA viruses. None of these compounds, however, would be considered "breakthroughs" in the sense of having dramatic antiviral effects. Indeed, the past two years might be considered relatively unproductive with regard to RNA virus inhibitors. It is hoped, however, that the materials reported herein will be of value as the synthesis of superior antivirals is considered.

Amantadine Derivatives - Among a series of sulfonylthiourea analogs of amantadine, 1 exhibited strong anti-influenza virus activity both in vitro and in vivo.[7] The activity compared closely with that of amantadine, which was tested in parallel. Of possible significance, 1 was better tolerated in mice than was amantadine. In a separate study,[8] a series of diphenylthioureas was active against polio, coxsackie B and echo viruses. Interestingly, compound 2--somewhat similar in structure to 1--showed the highest activity of all derivatives against these picornaviruses.

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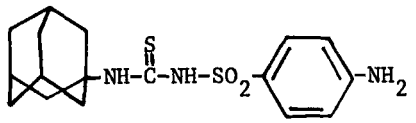

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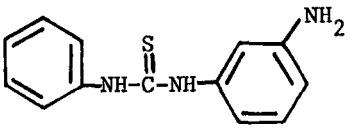
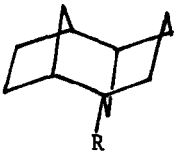


2

1-Substituted derivatives of tricyclo [4.3.1.1] undecane (3) have exhibited greater antiviral effects against Newcastle disease virus in vitro than amantadine.[9] The most active of these compounds were an amine hydrochloride, an ester, and an N-alkylamide.



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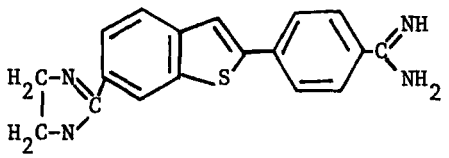
Amidines - A series of diarylamidine derivatives have a potent inhibitory effect on RNA-directed DNA polymerase (reverse transcriptase) of Moloney murine leukemia virus.[10] Most significant among these compounds were 4',6-diimidazolino-2-phenylbenzo[b]thiophene (4) and 5-amidino-2-(5-amidino-2-benzofuranyl)indole (5). These compounds were considerably more effective than the known[11] compound 4',6-diamidino-2-phenylindole. In preliminary experiments, a related symmetrical styryl diarylamidine was significantly active against the Moloney virus in mice.[10]



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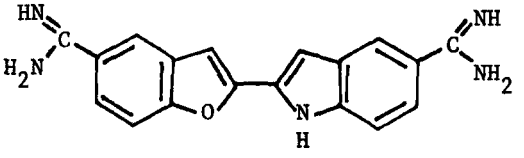

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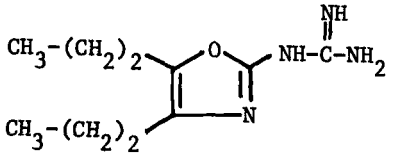
2-Aminoxazoles - The 2-aminoxazoles represent a possible new class of compounds having activity against RNA viruses. Three derivatives in this group, 2-guanidino-4,5-di-n-propyloxazole (6), 2-guanidino-4,5-diphenyloxazole (7), and the 2-amino analog of 7 had in vitro efficacy against coxsackie, polio and echo viruses.[12] The major effect appeared to be on inhibition of viral RNA synthesis, although some cellular RNA synthesis inhibition was also seen.[12]



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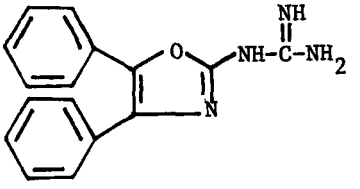
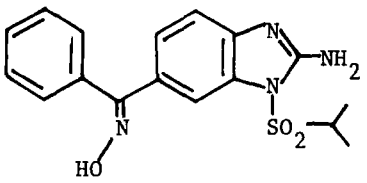


7

Benzimidazole Analogs - A number of benzimidazole analogs have exhibited rather broad spectrum antiviral activity, as reviewed elsewhere.[2] The recently synthesized syn and anti isomers of 6[[(hydroxyimino)-phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1H-benzimidazol-2-amine have significant in vitro activity against picornaviruses, particularly rhinovirus (all strains tested to date) coxsackie, echo and polio viruses.[13-16]

The compounds appeared to possess reasonably low toxicity and to be well absorbed orally based on their capacity to produce high blood and lung levels. Initial mechanistic studies indicate the compounds inhibit virus-directed macromolecule synthesis, probably through retarding assembly of the viral replication complex.[16] The anti isomer (8) was the more potent in comparative tests with many rhinovirus serotypes.[13] Another benzimidazole analog, bis(5-amidino-2-benzimidazolyl)methane (9) is an apparently specific inhibitor of respiratory syncytial virus. The compound appears to act by blocking viral penetration.[14]



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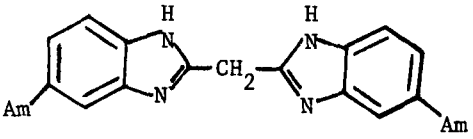
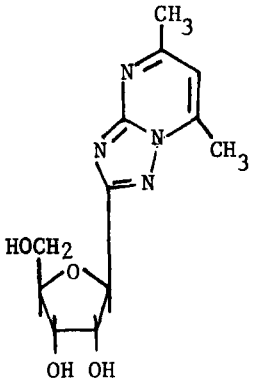


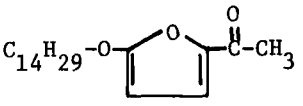
9

Bridgehead C-Nucleosides - Ribavirin, discussed in more detail below, and the related formycin A and B are known to inhibit RNA viruses to varying degrees.[2] A series of related C-nucleosides with a bridgehead nitrogen atom recently have been synthesized. In particular, 5,7-dimethyl-2-β-D-ribofuranosyl-s-triazole (1,5-a)pyrimidine (10), was inhibitory in vitro to mengovirus, an RNA virus closely related to poliovirus.[17] The compound reportedly did not affect cellular multiplication and synthesis of DNA or RNA in cultured cells under the same experimental conditions. The studies suggest that the flexibility of the glycosidic bond is important for determining the biological activity of such compounds.



10

1-(5-Tetradecyloxy-2-furanyl)ethanone - When tested in vitro, this compound (11) appears to have significant and specific activity against rhinoviruses.[18] Antiviral concentrations seem to be nontoxic for uninfected cells. Other RNA viruses (vesicular stomatitis, influenza A2, Semliki forest, polio type 2) and several DNA viruses were not affected. The primary action of the compound is apparently due to direct interaction between it and the virus capsid.



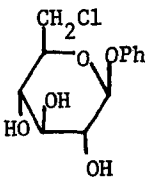
11

D,L-α-Difluoromethylornithine - This analog of spermidine affects the production of Semliki forest virus in cell culture.[19] Greatest efficacy was seen when cells were pretreated for 72 hours. The activity was reversed by addition of spermidine.

Glycyrrhizic Acid - A triterpenic component of Glycyrrhizia glabra roots, glycyrrhizic acid, has exhibited a rather broad spectrum of antiviral activity in cell culture studies.[20,21] RNA viruses inhibited included Newcastle disease virus and vesicular stomatitis virus. Poliovirus type

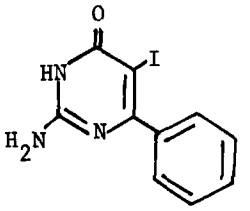
1 was not affected. The material appeared not to affect cell activity or cell replication. The mechanism of antiviral action of glycyrrhizic acid has not yet been determined.

Glycosides - Several sugar derivatives have been described as having significant antiviral activity. These include 2-deoxy-D-glucose, glucosamine, 2-deoxy-2-fluoro-D-mannose, and 6-amino-6-deoxy-D-glucose.[2] A series of phenyl glucoside derivatives have recently been synthesized, with phenyl-6-chloro-6-deoxy-β-D-glucopyranoside (12) and some related compounds exhibiting activity against influenza and herpesviruses. Later work with this compound showed it to be an inhibitor with particular specificity toward paramyxoviruses.[22,23] Cytotoxic effects were minimal at antiviral levels.



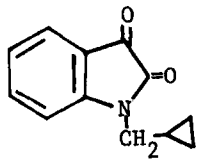
12

6-Arylpyrimidines - In a series of newly synthesized pyrimidines, 2-amino-5-bromo-6-phenyl-4-pyrimidinol and 2-amino-5-iodo-6-phenyl-4-pyrimidinol (13) were effective in mice against infections caused by Semliki forest and encephalomyocarditis viruses.[24] Both compounds were active whether administered by the intraperitoneal or subcutaneous routes. The bromo derivative was also active when given orally. The maximum tolerated doses of the compounds were 10 to 80 times greater than the antiviral doses, suggesting reasonable therapeutic indices. The bromo derivative and several related 6-arylpyrimidines were potent interferon inducers. In contrast, the iodo analog and several closely related derivatives possessed potent antiviral activity but did not induce interferon production. 5-Iodo-6-phenyl-pyrimidinone in particular had significant in vivo activity against both Semliki forest virus and the DNA-containing type 1 herpesvirus[25,26] but was a relatively poor interferon inducer.[26] This suggests a possible alternate and/or additional mechanism of antiviral action occurs within this series of compounds.



13

Isatin Derivatives - Thiosemicarbazone derivatives of isatin, such as methisazone, are widely recognized for their activity against DNA-containing pox viruses. A recent study confirmed that a small series of cyclopropane derivatives of methisazone also were active against a DNA virus (vaccinia).[27] Even though a thiosemicarbazide moiety is considered essential for activity, one of the new derivatives which is not a thiosemicarbazone--N-methylcyclopropylisatin (14)--had significant activity against parainfluenza type 3 virus.[27]



14

Antibiotics - A new antibiotic, virantmycin, has recently been isolated from the culture broth of a strain of Streptomyces nitrosporeus. It has significant inhibitory effect on vesicular stomatitis, sindbis, western equine encephalitis, and Newcastle disease viruses, as well as similar effects on the DNA-containing herpes and vaccinia viruses.[28]

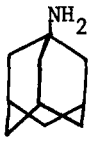
Oligopeptides - The peptide carbobenzoxy-phenylalanyl-phenylalanyl-nitroarginine has been shown to be a specific inhibitor of paramyxoviruses, particularly measles, SV5, and Sendai viruses.[29] Structure-activity studies with additional oligopeptides indicated an N-terminal phenylalanine was necessary for strong antiviral activity. The carbobenzoxy

group also enhanced activity. The results suggest that the activity is related to the similarity of these oligopeptides to the N-terminal region of a viral polypeptide.

ESTABLISHED INHIBITORS OF RNA VIRUSES

Despite the fact there may have been no "breakthroughs" in the chemotherapeutic control of RNA virus infections, there have been significant advances in the knowledge of how to use existing compounds and in understanding their mechanisms of action. The following section summarizes some of these recent findings.

Amantadine (1-adamantanamine, Symmetrel™) - Amantadine (15) was first approved by the FDA in 1966 for prophylactic use against Asian (H2N2) influenza. Based upon subsequent experience with the drug, approval was expanded in 1976 to permit its prophylactic use against all strains of influenza A. Comprehensive reviews of all aspects of the drug's development and use have been published recently by Oxford and Galbraith[30] and by Hoffmann.[5]



15

Initial studies on the mode of antiviral action of amantadine were interpreted as indicating the drug blocked penetration of the virus into cells or prevented its uncoating once in the cell.[6] More recent work has demonstrated, however, that influenza A virions penetrated cells and appeared in the cytoplasm despite the presence of amantadine.[30] Subsequent formation of viral polypeptides was inhibited;[30] as was the synthesis of virus-specific RNA.[31] Because the drug does not block the virion-associated RNA-dependent RNA polymerase,[30] amantadine does not appear to inhibit transcription of the viral genome directly. Recent work with amantadine-resistant viruses[32] suggests the possibility that amantadine interacts with the matrix protein to prevent uncoating of the virus or transcription of its genome.[30] Other mechanisms of drug resistance exist;[33] indicating additional biochemical actions of the drug are possible.

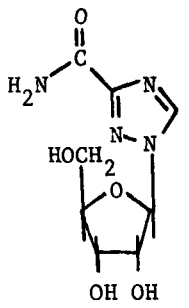
Pharmacological evaluations of amantadine have centered in recent years on the combination of the drug with other antiviral agents and on its administration by inhalation. Combinations of amantadine or the closely-related and clinically useful analog rimantadine[5,34] with ribavirin have been tested *in vitro*. Increased antiviral effects against four strains of influenza A were observed when ribavirin was combined with either of the other drugs.[35] Combination of ribavirin with a high concentration of rimantadine also was effective against a strain of influenza B.[35] In mice, the therapeutic use of amantadine reduced mortality from influenza A from 90% to 40% when the drug was administered by small-particle aerosol inhalation.[36,37] When combined with ribavirin, mortality was reduced even further to less than 10%. The intraperitoneal administration of amantadine was not effective but the combination with ribavirin did reduce mortality significantly.[36]

The efficacy of orally-administered amantadine for the prevention and treatment of influenza A in humans has been known for a number of years.[4] In 1979 a National Institutes of Health panel recommended that amantadine should be used for both prevention and therapy of influenza A in the next epidemic.[38] Additional clinical use and testing of the drug at the 200 mg/day oral dose has occurred during the past few years. Efficacy in one study was >70% in preventing serologically confirmed

Russian influenza A (H1N1). Side effects of the drug were mild and terminated on cessation of prophylaxis.[39] In a separate study, side effects were more significant than previously reported.[40] In another study, 300 mg/day rimantadine was better tolerated than amantadine due to central nervous system side effects caused by the latter drug.[41]

The administration of the drug to humans by small-particle aerosol inhalation has recently been explored. Inhalation of the drug by healthy individuals was generally well-tolerated based on pulmonary function tests.[42-44] Plasma and urine concentrations of amantadine demonstrated the drug was absorbed systemically.[42,43] Nasal wash concentrations exceeded plasma levels by greater than 4000-fold.[43] Limited clinical experience with this mode of administration to patients with influenza A virus infections[42,44] has demonstrated a more rapid resolution of clinical signs and symptoms compared to placebo-treated patients.[44]

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, Virazole™) - This synthetic, truncated nucleoside (16) inhibits the replication of both RNA and DNA viruses in vitro and in vivo. Many aspects of the drug's actions, pharmacology, toxicology and clinical efficacy have been presented in a new book edited by Smith and Kirkpatrick[45] and in a review by Sidwell and Olsen.[5]



16

The exact biochemical basis for the antiviral action of ribavirin is unknown. It is known, nonetheless, that the mode of action involves inhibition of nucleic acid biosynthesis.[6] Inhibition of DNA and RNA synthesis apparently is caused by nucleotide metabolites of ribavirin inhibiting IMP dehydrogenase, mRNA guanylyltransferase and/or influenza virus RNA polymerase.[1,6] The fact that ribavirin must be converted to the nucleotide level to be active was substantiated recently by the observation that the inactive 2'- and 3'-O-methyl derivatives of ribavirin were not phosphorylated by a nucleoside kinase preparation which readily phosphorylated ribavirin and adenosine.[46] Neither compound possessed antiviral activity nor inhibited DNA synthesis. The ready phosphorylation of ribavirin by adenosine kinase[6] may be related to the molecular conformation of the drug.[47]

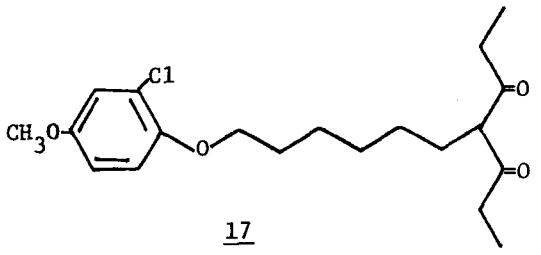
The antiviral activity of ribavirin in vitro has been explored with additional viruses. The drug was active against isolates of the slow virus, subacute sclerosing panencephalitis virus,[2,48] and against respiratory syncytial virus[49]--the virus responsible for pneumonia and other respiratory diseases seen primarily in young children. In addition, ribavirin was active in cell culture against two plant viruses, tomato white necrosis virus[50] and tomato spotted wilt virus.[51]

The administration of ribavirin by small-particle aerosol inhalation to influenza A-infected mice produced dramatic cure rates.[36,37,52] The therapeutic use of ribavirin in this manner was superior to that of either amantadine[36,37] or rimantadine.[52] The combination of ribavirin with amantadine was better than either drug alone.[36,37] When administered by inhalation, ribavirin was effective in mice infected with influenza B whereas amantadine was not.[36] Ribavirin administered by the subcutaneous route also was effective against Lassa fever virus in experimentally infected rhesus monkeys.[53] A clinical trial of the drug against Lassa virus patients is underway in Sierra Leone.[54]

Additional information on the toxicology of the drug was published during the last year.[45,55-58] Ribavirin was teratogenic to mice at doses of >25 mg/kg.[55] The drug appeared to inhibit embryonic DNA synthesis both in vitro and in vivo. Ribavirin inhibited lymphocyte proliferative responses as measured by [^{3}H]thymidine and [^{3}H]uridine incorporation.[56] Other work has established, however, that [^{3}H]thymidine incorporation does not accurately measure DNA synthesis in human cells treated with ribavirin.[45,57] The drug also affected tolerance induction in mice with rabbit gamma globulin.[58]

A recent clinical trial with ribavirin against a naturally occurring outbreak of influenza A (H1N1) failed to alter clinical signs and symptoms of the disease.[59] The 1000 mg/day oral dose of drug also did not alter viral shedding but did appear to limit rises in antibody titer to influenza A viral antigen assayed by complement fixation.[59] Inadequate drug distribution to the respiratory tract following divided oral doses may be responsible for the variable clinical efficacy of ribavirin against influenza.

Arildone (4-[6-(2-chloro-4-methoxy)phenoxy]hexyl-3,5-heptanedione, Win 38020) - Arildone is one of a series of β-diketones that are active against DNA and RNA viruses.[1] Recent work has demonstrated arildone (17) has a very potent effect on the in vitro replication of polio virus type 2 (99% inhibition at 3 μg/ml).[60] This concentration was less effective (90 to 99% inhibition) against vesicular stomatitis virus, coxsackie virus A9, and the DNA viruses herpes simplex types 1 and 2. Lesser effects were observed against other DNA viruses and Semliki forest virus. At 3 μg/ml arildone had minimal effects on the growth of several mammalian cell lines. A concentration of 8 μg/ml, however, inhibited cell growth and significantly reduced the incorporation of labeled precursors into acid precipitable material.[60]



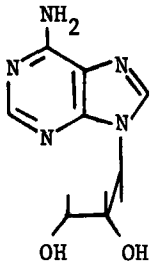
17

Like ribavirin, arildone is not virucidal and does not induce interferon production in cultured cells or in mice.[1] Arildone also does not interfere with adsorption or penetration of polioviruses into cells.[1] It does, however, prevent the uncoating of poliovirus in the infected cell and thereby prevents virus-induced blockage of host cell protein synthesis. Virion uncoating appears to be blocked by a direct but weak interaction of the drug with the viral capsid.[61]

In experimental animal infections, arildone has been effective when applied topically.[1] Recent results indicate that an antiviral agent related to arildone, diethyl-6-[2-chloro-4-methoxy)phenoxy]hexyl phosphonate (Win 42202) is active topically against herpesvirus infections.[62]

(S)-9-(2,3-Dihydroxypropyl)adenine [(S)-DHPA] - (S)-DHPA (18) is one of two antiviral nucleosides in which the sugar is replaced by an aliphatic side chain.[1] Unlike acyclovir, whose reported activity is restricted to herpesviruses,[1] (S)-DHPA is active against both DNA and RNA viruses at concentrations that do not adversely affect cellular functions.[63] (S)-DHPA also inhibits transformation of chick embryo fibroblasts by Rous sarcoma virus (RSV), an oncornavirus.[64] Inhibition was reversible suggesting

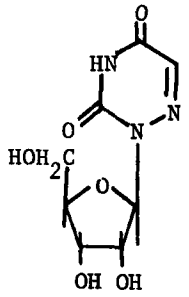
the compound did not affect integration of proviral DNA into the cellular genome.[64] Although the antiviral mode of action of (S)-DHPA is unknown,[63] preliminary data on cell transformation open the possibility that (S)-DHPA may inhibit the virus-specified protein kinase known to be involved in oncogenic transformation.[64] In addition, (S)-DHPA is an inhibitor of adenosine deaminase.[63]



18

In a recent study,[65] the potency of (S)-DHPA against vaccinia virus (a DNA virus) was increased markedly by combining it with 6-azauridine (19). The combination was active to a lesser extent against two RNA viruses, Newcastle disease virus and western equine encephalomyelitis virus.[65] In preliminary animal work, (S)-DHPA reduced by one-half mortality caused by intranasal inoculation of mice with vesicular stomatitis virus.[63] The metabolic disposition of [8-^{14}C](S)-DHPA has been examined in mice.[66] A significant amount of the dose accumulated in the liver and was converted to an unidentified metabolite. Unchanged drug was rapidly excreted in the urine.

6-Azauridine (z^6Urd) - 6-Azauridine (19) is active against several RNA and DNA viruses but with considerable differences in potency. For example, 6-azauridine is a very potent inhibitor of dengue virus but is only a weak inhibitor of herpesviruses. A thorough review of the antiviral spectrum of 6-azauridine and all aspects of the compound's chemistry, biology and clinical usefulness has been published recently.[67]



19

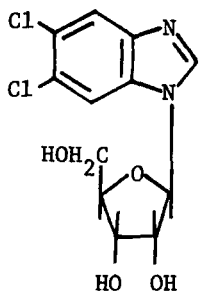
As with other nucleosides, 6-azauridine must be converted to a nucleotide to be active. 6-Azauridine is phosphorylated by uridine kinase to the 5'-monophosphate (z^6UMP) which acts as a competitive inhibitor of orotidylate (OMP) decarboxylase. Inhibition probably is a consequence of conformational similarities between z^6UMP and OMP.[68] Inhibition of OMP decarboxylase blocks the formation of UMP and subsequent synthesis of RNA and DNA. Selective inhibition of virus replication, where observed, may arise as a consequence of the capacity of some viruses to increase the activity of uridine kinase, thereby creating high concentrations of z^6UMP in virus-infected cells.[67]

The antiviral activity of the compound has been explored in several animal models and in humans. Despite the fact that 6-azauridine was not highly active against pox and herpesviruses in vitro, most experimental animal and human studies have concentrated on these DNA viruses. Limited experience with herpes keratitis in rabbits and humans indicates the possibility of beneficial effects. Success in other studies was variable.[67] Although large doses of the drug to experimental animals and humans produced no serious acute toxicity, the drug is immunosuppressive and teratogenic.[67,69] Thus, potential antiviral usefulness in humans is limited by low potency and possible toxicity.[69]

Interferons - A review of interferons is beyond the scope of this article. Nonetheless, it should be mentioned that recent advances in large scale production of human interferons by leukocytes and by bacteria containing recombinant DNA should make extensive clinical trials possible. Substantial quantities of interferon are needed for antiviral clinical trials alone because different types of human interferon have been recognized

and various viruses respond differently to these types of interferon.[70] The reader is referred to Chapter 21 and to recent reviews by Stewart and Lin,[71] Krim et al.,[72] and Gregoriadis[73] for information on the production of interferon,[72] its antiviral and antitumor activity[71,72] and its targeting to specific sites.[73]

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) - The effects of DRB (20) and other ribosyl halobenzimidazoles on cellular and viral RNA synthesis have been reviewed extensively by Tamm and Sehgal.[74,75] DRB is a reversible, potent inhibitor of RNA synthesis in mammalian cells. Heterogeneous nuclear RNA, cytoplasmic messenger RNA and 45 S ribosomal RNA are inhibited most severely.[74] Recent data have shown that DRB acts by increasing premature chain termination of early[76] and late[77] adenovirus RNA and of cellular RNA synthesized by RNA polymerases I[78] and II[75,79] but not RNA polymerase III.[78] Inhibition does not appear to be dependent upon the minute amounts of DRB triphosphate which are formed in treated cells.[78] These and other results[79,80] indicate that DRB does not act as a competing nucleotide analog and suggest that inhibition is most potent against RNA that is extensively transcribed from genomes containing extensive intervening sequences.[78]



20

DRB is a potent inhibitor of several RNA viruses, including influenza virus.[74] DRB also has the interesting property of increasing interferon production in cells induced by poly(I)·poly(C).[74] DRB has not been tested extensively as an antiviral drug possibly because of its potent effects on RNA synthesis in uninfected cells. Cytotoxic drug levels, however, do exceed levels needed to inhibit the replication of some viruses thereby suggesting antiviral potential.[74] Furthermore, human diploid fibroblasts recover from exposure to DRB, whereas at least one neoplastic cell line does not. This raises the possibility that DRB and its congeners may possess antineoplastic activity.[74]

Analogs of DRB which are not nucleosides, such as 2-(α-hydroxybenzyl)-benzimidazole (HBB) and related compounds, are selective and reversible inhibitors of the replication of many picornaviruses, including polio and coxsackie viruses.[75] HBB inhibits viral RNA synthesis at concentrations that do not appear to affect cellular functions. One stereoisomer of HBB has shown protective activity in experimental enterovirus infections in an animal model.[75]

Other Inhibitors - Two compounds generally thought of as inhibitors of DNA virus replication also are active against RNA viruses. Phosphonoformate (PF)[1] is an inhibitor of RNA-directed DNA polymerases (reverse transcriptase) from several viruses[81,82] and therefore inhibits the replication of an RNA virus.[81] Glycosylation inhibitors, such as 2-deoxy-D-glucose, inhibit the replication of many enveloped viruses,[83,84] not just the DNA-containing herpesviruses.[1] Inhibition is not apparent, however, when glucose is present in culture medium.[83]

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Chapter 16. Veterinary Drugs

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Introduction - The search for new veterinary drugs involves numerous investigators using similar methodology and in many ways complementing investigations on new human drugs. For example, the 1980/81 edition of Veterinary Pharmaceuticals and Biologicals[1] lists more than 1600 veterinary products. Most drugs developed for human use eventually find a place in veterinary medicine, but it was decided to restrict this review to drugs developed solely for veterinary use or drugs which are used much more extensively in veterinary medicine than in human medicine. For this first report, an attempt has been made to describe recent advances and to place these advances in perspective relative to standard veterinary practice. An interesting article appeared on the cost of developing new veterinary drugs.[2]

Protozoal Diseases

Coccidiosis - Coccidiosis continues to attract much attention from industrial and academic researchers. Various aspects of chemotherapy,[3] development of resistance,[4] and screening and evaluation of drugs[5] were reviewed. Thymidylate synthetase was demonstrated in unsporulated oocysts of E. tenella, and its inhibition was suggested as a target for chemotherapy, although the parasite enzyme is not substantially different from that of the chicken host.[6] Other enzymes proposed as targets for chemotherapy of protozoan parasitic infections via inhibition of nucleic acid syntheses are orotate phosphoribosyltransferase and orotidine-5-monophosphate decarboxylases (Leishmania mexicana and Crithidia fasciculata),[7] 5-adenosylmethionine-dependent methyltransferases (P. falciparum)[8] or hypoxanthineguanine phosphoribosyl transferases.[9] The controversy over the mode of anticoccidial action of arprinocid (1) appears to be settled in favor of an active 1-N-oxide metabolite, which does not inhibit hypoxanthine transport but binds to the heme moiety of cytochrome P-450 in microsomes.[10,11] Although eight years of intensive use of monensin (2) in broiler production were remarkably free of drug-resistant coccidia, E. meleagrimitis strains resistant toward several ionophores were demonstrated for the first time in turkeys.[12] Some concern was expressed over monensin residues in meat and their possible adverse effects on heart and blood vessels of sensitive cardiac patients.[13] Comparative field trials were reported on arprinocid (1)[14,15] and halofuginone (3),[16,17] and the ionophores lasalocid (4),[18,19] salinomycin (5)[20] and narasin (6),[21] in chickens, turkeys or calves. Analogs of arprinocid with alkyl substituents on the 6-amino group were reported.[22,23] The mono- and dimethylamino derivatives showed the highest potency; further chain elongation was detrimental to activity, suggesting a possible metabolic demethylation to arprinocid as the active principle. The 6-azauracil anticoccidials substituted at N-1 with, for instance, 3,5-dichlorophenyl (7) have unfortunately high plasma levels and long half-life, a distinctly undesirable property for a drug intended for use in food animals.[24] Further development of this lead resulted in a highly potent derivative with a 3,5-dimethyl-4-(p-chlorophenylthio)-phenyl substituent at N-1 (8) having a shorter plasma half-life, possibly due to rapid in vivo oxidation of the sulfide to the sulfone.[25] Introduction of a sulfonamido group into the highly lipophilic molecule resulted in a significant reduction of plasma drug levels and half-life times

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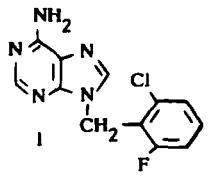
accompanied by only a modest loss in coccidiostatic activity, with 1-(3,5-dimethyl-4-(morpholinylsulfonyl))-6-azauracil (9) being fully effective against a spectrum of chicken Eimeria species at 50 ppm in feed.[26] Substitution of a sulfonamido group for the carboxamide of 5-nitronicotinamide resulted in a moderate increase of activity against E. tenella.[27] Certain acridine derivatives (10) having activity against E. tenella and P. berghei[28] and two naphthoquinone (11, 12) antibiotics with potent E. tenella activity[29] were described. The discovery of new ionophores continues.[30] Synergistic activity against E. tenella was described for a combination of t-butylaminoethanol and sulfaquinoxaline.[31]



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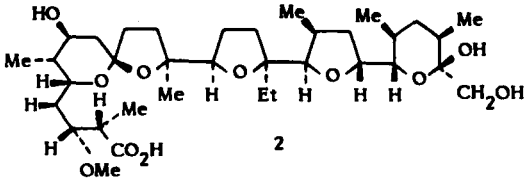

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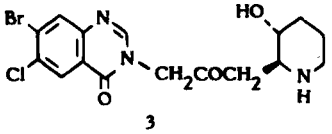

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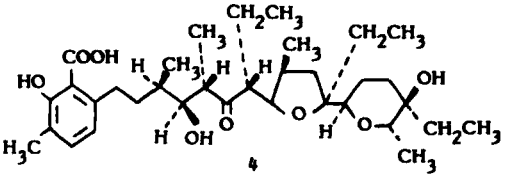

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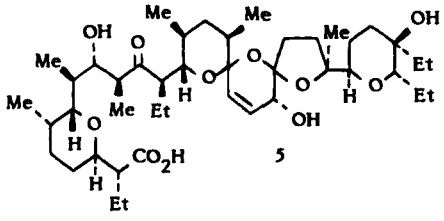

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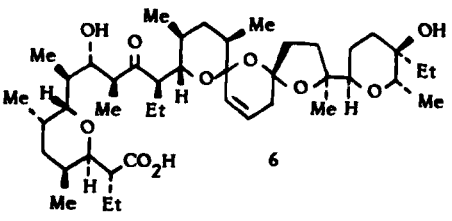

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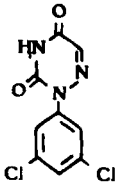

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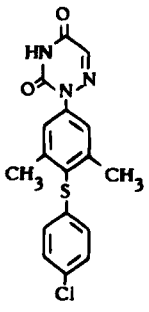

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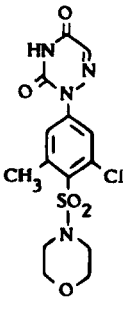

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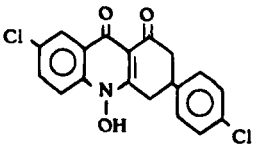

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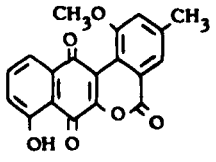

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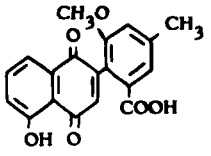

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Trypanosomiasis - A WHO publication reviewed the status of human and animal trypanosomiasis of the last ten years. About 25 million cattle are currently afflicted with the disease, and large areas of the African continent are completely unable to sustain livestock due to trypanosomiasis.[32] A report of a workshop on the application of recombinant DNA technology to protozoans, especially trypanosomes, has appeared.[33] The uptake of purine bases by T. brucei and T. congolense was investigated; the design of inhibitors of purine uptake was advanced as a target for chemotherapy, although a few naturally-occurring

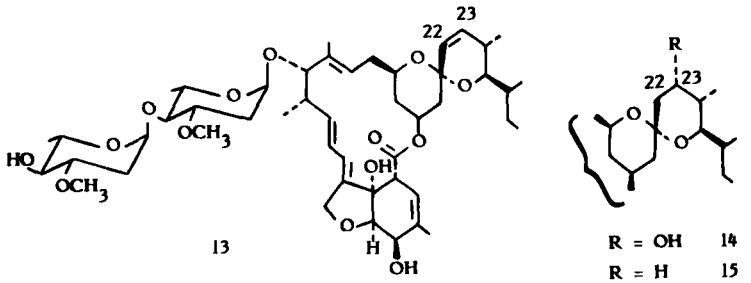
nucleoside antibiotics proved disappointing as trypanocides.[34] Administration of α-difluoromethylornithine in drinking water eliminated T. brucei infections from mice. This highly specific ornithine decarboxylase inhibitor blocks polyamine synthesis, which is essential for the growth of rapidly proliferating cells. Polyamine metabolism is proposed as a chemotherapeutic target.[35] The need for effective new antitrypanosomal drugs was again stated, and a partially automated in vitro screening method based on the uptake of (^{3}H) thymidine and (^{14}C) leucine by T. rhodesiense was described in which aromatic diamidines, carbamoyl dithioates, 2-acetylpyridine thiosemicarbazones[36,37] and allopurinol were found active, but not tryparsamide and suramin.[36] A combination treatment with diminazene aceturate and a nitroimidazole was able to cure otherwise relapsing T. brucei infections in mice.[38,39] A number of bis(4-guanylphenyl)-substituted oxazoles were shown to have activity against T. rhodesiense in mice.[40] Twenty-five compounds were found to have some degree of activity against T. rhodesiense in mice in a screening program.[41]

Protozoal Diseases of Minor Importance - Veterinary protozoal infections of currently limited interest to medicinal chemists are babesia in cattle and horses and histomoniasis in turkeys for which imidocarb and dimetridazole, respectively, are available for therapy.[42] Menoctone was reported as an effective treatment of theilerosis of cattle,[43] and amprolium was shown to have prophylactic activity against ovine sarcosystis.[44]

Helminth Diseases

General - A new edition of an encyclopedic book covering the biochemistry and physiology of parasitic helminths and protozoans with literature coverage to 1977 has appeared,[45] and a useful text on the physiology of these parasites was published.[46] Currently accepted chemotherapy of helminthiasis in cattle,[47,48] sheep,[49] horses,[50] and dogs and cats[51] was reviewed.

Nematodes - In several experiments, avermectin $B_{1}a$ (C-076 $B_{1}a$), **13**, was found completely effective at 0.1-0.2 mg/kg against essentially all mature and larval forms (including inhibited stages) of gastrointestinal and systemic nematodes, (including benzimidazole-resistant strains) in sheep[52-54] and cattle.[52-56] Avermectin $B_{1}a$ also showed excellent activity against common equine nematodes including Strongylus vulgaris (adult[57] and migrating larvae[58]) and Onchocerca spiralis.[48] Avermectin $B_{2}a$ (**14**) at 0.3 mg/kg reduced by 85% the number of Trichinella spiralis in mice[59] and at 0.05 mg/kg was highly effective against circulating microfilariae, but not adult, Dirofilaria immitis in dogs.[60] Avermectin $B_{1}a$ apparently acts by selectively interfering with GABA-mediated neurotransmission in invertebrates.[61,62] Ivermectin (22,23-dihydroavermectin B_{1}, MK-933, **15**), prepared by selective hydrogenation of **13**,[63] was found equal or superior to **13** against parasitic nematodes of sheep,[64] cattle,[65-67] horses,[68,69] and swine,[70] as well as against microfilariae of D. immitis in dogs[64] and ferrets,[71] and is under development as a new veterinary antiparasitic agent.[63] The structure-activity relationships of analogs of **13** and **15** were also discussed.[63]



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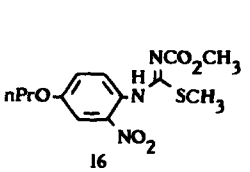
R = OH 14

R = H 15

Febantel was found active against common nematodes of horses,[72] sheep[73] and dogs,[74] but reports of cross resistance with benzimidazole resistant species appeared.[71-73] A critical evaluation of tioxidazole in horses revealed generally good activity, but benzimidazole resistant parasites were refractory.[76,78] A pharmacokinetic study of albendazole in sheep showed that the corresponding sulfoxide and sulfone are probably responsible for much of this drug's activity.[79] Fenbendazole was reported highly effective at 50 mg/kg against Ancyclostoma caninum and Toxocaris canis in dogs.[80] Mebendazole was found active against Litomosoides carinii in the cotton rat[81] and 4-week-old T. spiralis in mice.[82] It was shown that Brugia pahangi does not incorporate exogenous thymidine,[83] suggesting that inhibition of de novo pyrimidine biosynthesis may be of chemotherapeutic use, while studies of B. pahangi and D. immitis suggested that 5,10-methylenetetrahydrofolate may also be a target for chemotherapy.[84,85] Levamisole was found effective against both adult D. immitis and microfilariae in dogs.[86] Hydroxyethyl starch was found useful for cryopreservation of microfilariae,[87] and methods of testing substances for antifilarial activity were reviewed.[88] A new in vitro test for potential anthelmintics employing Caenorhabditis elegans was reported.[89] The phenylisothiourea derivative **16** (Sch 23154) was found active against Syphacia obvelata.[90] Structures that appeared in the patent literature with claims of nematocidal activity were phenylfuran (**17**),[91] dithiocarbamate (**18**)[92] and benzimidazole (**19**),[93] while **20** and twelve analogs were claimed to have good activity against hookworm.



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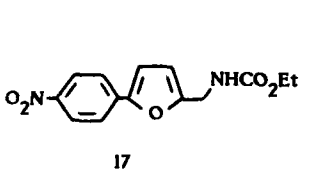

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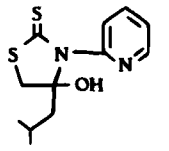

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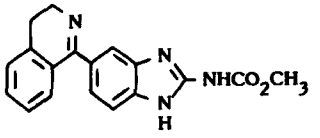

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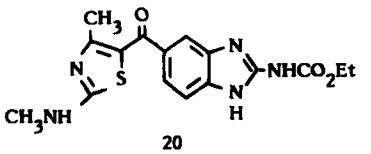

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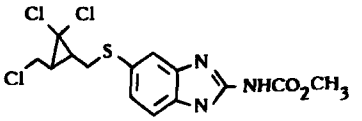
Cestodes - Praziquantel at 50-100 mg/kg in calves completely eliminated viable cysts (Cystercercis bovis) of Taenia saginata,[94-96] and at lower doses was effective against Echinococcus multilocularis and other cestodes in dogs and cats.[97] Albendazole was found effective against Thysanosama actinoides in sheep.[98] Antibiotic S15-1 (SQ 21,704), a complex belonging to the streptothricin family, was found highly active against T. pisiformis, T. taeniaeformis, Diphyllobothrium caninum and Spirometra mansonoides in dogs and cats.[99] Compounds with cestocidal activity appearing in the patent literature are **21**[100] (also active against whipworm), **22**,[101] and several phosphoureides.[102,103]



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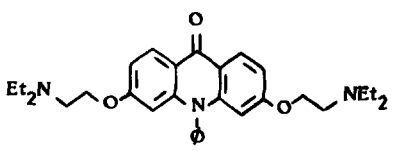

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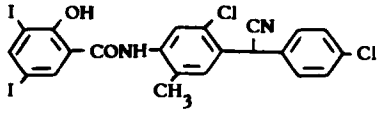
Trematodes - Closantel (**23**), a new salicylanilide derivative, was found effective at 5-10 mg/kg in sheep against mature and immature Fasciola hepatica[104] and benzimidazole resistant Haemonchus contortus[105] but not Trichostrongylus colubriformis.[105] Salicylanilide **24** was highly potent against immature F. hepatica

in sheep (0.2 mg/kg p.o.) but was toxic at ≥ 1.5 mg/kg.[106] The disulfonamide **25** inhibits fasciolar 3-phosphoglycerate kinase and phosphoglycerate mutase in vitro[107] and binds tightly to rat erythrocyte carbonic anhydrase in vivo[108] suggesting that **25** enters the fluke at least in part by ingestion and acts by



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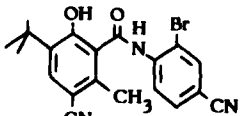

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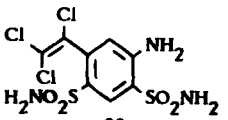

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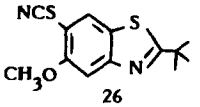
inhibiting glycolysis.[107,108] The importance of glucose as an energy source for liver flukes in vivo has been questioned, however.[109] Single doses of 10-15 mg/kg of albendazole were effective against F. hepatica[110-112] and F. magna[111] in cattle, and in multiple doses, against F. hepatica in sheep.[113,114] Albendazole was also found active against Dicrocoelium dendriticum[115] and D. lanceolatum[116] in sheep, while fenbendazole showed good activity against Echinostoma caproni in mice[117] and modest activity against Liorchis scotae in cattle.[118] Phenacetin at 150 mg/kg for two days was ≥ 90% effective in controlling immature F. hepatica in sheep.[119] A protein isolated from the mammary gland of cows infected with F. hepatica was able to destroy juvenile F. hepatica at concentrations of $< 10^{-6}$M in vitro.[120] Other fasciolicides claimed in the patent literature are **26**[121] and **27**[122] (also active against cestodes and nematodes).



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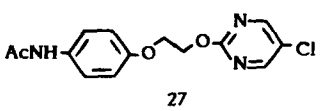

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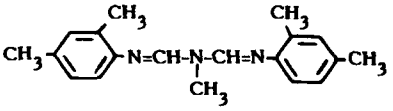
Ectoparasites

Arthropod pests have long been recognized to cause major economic losses in livestock production. A recent review[123] points out 45 species of arthropods parasitizing cattle in the United States. The list includes lice, flies, ticks and mites. Most of the compounds used for ectoparasite control were originally developed as agricultural insecticides and later found uses as animal dips or sprays. Synthetic pyrethroids[124-126] and organophosphates[127,128] are widely used, although resistance has been reported to diazinon[129] and chlorinated hydrocarbons[130] in flies and ticks.

Amitraz (**28**) was shown to be effective against mange mites in pigs.[131] The potentially most important advance in ectoparasite control was the announcement that ivermectin (**15**) was highly effective against mites,[132,133] ticks and larvae of biting flies,[64] by topical or systemic application. This discovery makes ivermectin the first compound to show systemic broad spectrum, anthelmintic and ectoparasitic activity.



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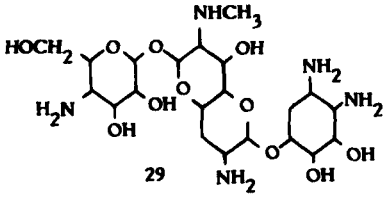
Bacterial Infections

Animal bacterial infections where chemotherapy is showing some effectiveness are swine dysentery, principally caused by Treponema hyodysenteriae, mycoplasmal pneumonia, colibacillosis, caused by enterotoxigenic Escherichia coli, and mastitis.

Apramycin (29), an aminocyclitol antibiotic, was shown to control neonatal colibacillosis in pigs by oral,[134] parenteral[135,136] or administration in feed.[137] Diarrhea was eliminated, and mortality was reduced in calves treated with cephamycin C (30) after experimental E. coli infection.[138]



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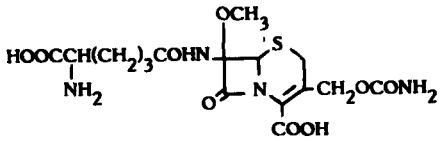

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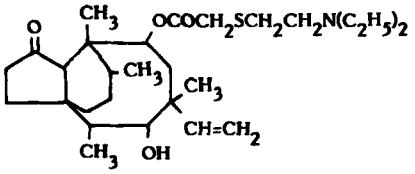
A review of swine dysentery was published.[139] Tiamulin (31), a semi-synthetic derivative of pleuromutilin, was shown to be effective in controlling experimental swine dysentery by administration in drinking water or in feed. There may have been some effect against enzootic pneumonia at the highest levels tested.[140] Previous studies also indicated activity against naturally acquired mycoplasmal pneumonia in pigs by administration of tiamulin in drinking water.[141] Other studies indicated that the antibiotic lincomycin,[142] the quinoxalines carbadox[142] or mecadox,[143] and the nitroimidazoles ronidazole,[142] dimetridazole[144] or ipronidazole,[145] had a beneficial effect on swine dysentery.



31

There is still a need for agents to effectively control mycoplasmal pneumonia in cattle, sheep, swine and poultry. A recent study indicated that ronidazole, oxytetracycline and tylosin had a beneficial effect on the severity of transmission of mycoplasmal pneumonia in sheep. Somewhat surprisingly, penicillin markedly suppressed the development of lesions, indicating bacterial involvement.[146] A paper appeared describing the in vitro susceptibility of Mycoplasma hyopneumoniae and Mycoplasma hyorhinis to 51 antimicrobial agents.[147]

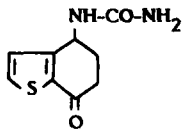
Several reviews appeared describing mastitis in dairy cattle and focused on control,[148] economic losses[149] and bacteriology.[150] The disease is of complex bacterial etiology, is poorly controlled and is responsible for large economic losses in dairy cows. A paper was published describing the in vitro activity of alkyl amines against mastitic bacteria.[151]

Lactic Acidosis - Lactic acidosis in ruminants, primarily in cattle, is generally a consequence of attempts to optimize growth and feed efficiency in feedlots. When cattle or sheep are abruptly changed from roughage to a concentrate diet, Streptococcus bovis, present in the rumen, proliferates causing an overproduction of lactic acid resulting in illness and sometimes death. Management techniques and antacids are used to alleviate the condition. Recently, a search was undertaken to find a potent selective antibiotic to control S. bovis[152] and, hence, the condition. Investigations in sheep indicated that thiopeptin at low levels in the diet effectively reduced rumen lactate 80-90%.[153,154]

Growth Promotion and Feed Efficiency

Compounds which promote growth in food animals may be divided into two types. Growth permittants are antimicrobial drugs with beneficial effects on gut flora and intestinal absorption. Growth stimulants improve regulatory mechanisms.

Growth Stimulants - Estrogens in general are known to stimulate growth in ruminants. However, diethylstilbestrol, the most widely used estrogenic growth stimulant in cattle, was recently prohibited from use in food animals,[155] due to concern for its carcinogenic potential. An extremely interesting new growth stimulant, sulbenox (**32**), was reported to stimulate growth and feed efficiency in rats and wether lambs.[156] Its mode of action is unknown, but it is not estrogenic.



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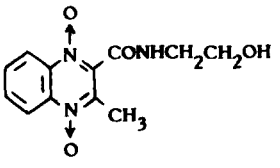
Growth Permittants - Antibiotics, such as bacitracin, penicillin, bambermycin, tylosin, virginiamycin and tetracyclines, and synthetic antibacterials, such as furazolidone, carbadox and sulfathiazole, are routinely mixed in animal feed at low levels to aid growth. There is a growing attempt to supplant these drugs with newer compounds structurally and mechanistically unrelated to antibiotics used in human medicine, because of concerns about resistance transfer. Two related sulfur-containing peptide antibiotics with these characteristics were recently described. Thiopeptin substantially increased rate of gain and feedlot efficiency in feedlot steers.[157] Nosiheptide was shown to be an effective, probably non-absorbed, growth permittant and feed efficiency enhancer in pigs[158] and broiler chickens.[159,160] Avoparcin, a glycopeptide antibiotic of the ristamycin, ristocetin type, gave mixed results in broiler chickens[161] and cattle,[162,163] in some instances improving gain and efficiency but in other cases having no significant effect. Olaquindox (**33**) increased rate of gain significantly in pigs.[164]



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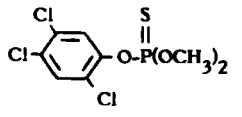
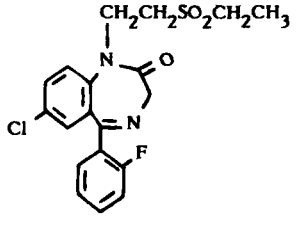
Feed Efficiency - Optimization of feed efficiency is a major goal in raising animals for food. Ionophores, such as lasalocid,[169] monensin[166-169] and narasin,[170] increased feed efficiency in cattle 5-10%, presumably by causing a favorable shift in volatile fatty acid synthesis in the rumen. Avoparcin showed a similar effect[162] distinct from its growth permittant properties. O,O-Dimethyl-O-(2,4,5-trichlorophenyl)phosphorothioate (RONNEL[R] **34**), a well-known insecticidal acetylcholinesterase inhibitor, had a positive effect on growth and feed efficiency in cattle unrelated to any reduction of insect or parasite burdens.[171] The effect was not explained by changes in rumen biochemistry or plasma amino acid patterns.[172] The finding is most surprising, and further investigation is needed.



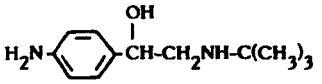
34

Feed Stimulants - Stimulation of feed intake is another possible way to increase productivity of food animals, and many compounds have been tried without real success. Elfazepam (**35**) increased feed intake in both sheep and cattle but gave variable responses in feed efficiency.[173]



35

Estrus Regulation and Fertility - Scheduling of reproductive events to fit a predetermined plan is now a standard management practice in the raising of livestock. Publications appeared on the use of a synthetic progestogen 17-α-allylestratriene-4,9,11,17-β-ol-3-one, (RU2267, REGUMATE[R]) for estrus synchronization of pigs[174-176] and of two prostaglandin $F_{2\alpha}$ analogs, ONO-1052[177] and ICI 80996 (cloprostenol), in cattle[178,179] and goats.[180] Postponement of parturition was achieved in cattle using the highly selective β_2 adrenergic agonist (**36**) (PLANIPART[R]).



36

A review of the veterinary use of synthetic antiviral agents was published.[182]

Unsolved problems still in the research stage dealt with the use of 1,25-dihydroxycholecalciferol in parturient paresis in cattle,[183] 3-methylindole and its involvement in pulmonary disease in cattle,[184] chemical defleecing of sheep,[185,186] the identification of methyl p-hydroxybenzoate in vaginal secretion of bitches in estrus and its possible role as a sex attractant,[187] and E. coli enterotoxins.[188]

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Section IV - Metabolic Diseases and Endocrine Function

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Chapter 17. Diabetes Mellitus

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Research on the treatment of diabetes mellitus has centered on attempts to achieve normal fasting and postprandial blood-glucose concentrations by exogenous-insulin, oral-drug, and dietary therapies. Tight control of blood-glucose levels in diabetics may counteract the development of complications. A recent long-term study, involving 4400 patients, concluded that the incidence of neuropathy and microangiopathy directly correlated with poor control of blood glucose.[1] However, more studies of well-controlled diabetics are needed to assess accurately the role of high blood-glucose levels in diabetic complications. Since lowering of blood sugar alone may not prevent the chronic complications of diabetes, research has also focused on correction of their underlying metabolic abnormalities.[2-5] Inhibitors of aldose reductase (AR) are being pursued because high, localized concentrations of sorbitol are believed to cause cataracts, retinopathies, and neuropathies.[2,3,6]

Comprehensive reviews on blood-glucose regulation,[7] and chronic complications of diabetes and AR inhibitors,[6] cover literature up to early 1977. This chapter covers literature on diabetes mellitus from 1977-1980.

Classification and Diagnoses of Diabetes - In 1979, the National Diabetes Data Group (NDDG) of the NIH, in collaboration with several national diabetes organizations and the WHO, proposed a detailed diabetes classification system.[8] The NDDG also appealed for world-wide standardization of diagnoses, terminology, and the oral glucose-tolerance test. Classification of diabetics involves their assignment to either of two main groups: (1) insulin-dependent, ketosis-prone (Type I; IDD) [also juvenile-onset (JOD)] and (2) non-insulin-dependent, non-ketosis-prone (Type II; NIDD) [also maturity-onset (MOD)]. The terms JOD and MOD are not recommended because they wrongly imply restriction to specific age groups.

Measurement of hemoglobin A_{1c} (HbA_{1c}) has gained popularity as a means for assessing long-term blood-sugar control in diabetics.[9] High HbA_{1c} levels reflect duration and severity of hyperglycemia; however, since HbA_{1c} turnover is slow, the use of HbA_{1c} measurements for establishing dosage regimens of insulin and oral hypoglycemic agents is impeded.[2,3,10]

Genetic and Etiological Factors - Lucid reviews[4,11] on these subjects appeared in 1979. Studies on NIDD identical twins showed that incidence of NIDD is largely influenced by genetics. NIDD twins may not develop diabetes simultaneously; however, they usually become concordant within ca. five years. About 65% of the general NIDD population demonstrate chlorpropamide-alcohol flushing (CPAF), which is also genetically determined.

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CPAF is caused by an increased sensitivity to enkephalin.[11] Monozygotic IDD twins show about 50% discordance, which suggests that IDD may not be entirely associated with genetic causes. IDD may be induced by "triggers", such as viruses and chemotactic agents, which cause autoimmune responses toward pancreatic β-cells.[4] Frequent discordance among identical twins, one IDD and one nondiabetic, may reflect differences in exposure to these "triggers". Certain viral infections are either known or suspected to cause IDD in humans and genetically susceptible members within a given strain of mice.[4]

Genes coding for the human leukocyte antigen (HLA) system are found at 4 loci on chromosome 6.[4] An individual may have two different alleles at a given locus, one derived from each parent. Both alleles, expressed as cell-surface proteins in the HLA system, can be identified and used for tissue-typing prior to transplantation and for indicating predisposition of individuals to certain diseases. These latter observations have opened a vast area of research concerned with correlating diabetics with HLA types in a wide variety of human models.[12-19] Results indicate that certain HLA types correlate additively with increased incidence of IDD,[19-21] while other types may correlate with decreased incidence.[21]

Dietary and Nutrition Therapy - Obese NIDDs often show resistance to the actions of endogenous insulin. Weight loss of only 10-15% improves insulin responsiveness and, thereby, glucose tolerance.[5] Recently, established dietary concepts for diabetics have been challenged.[5] Diets high in protein, once thought desirable for diabetics, may be deleterious. In mild IDDs, diets high in complex carbohydrates and fiber effectively lower insulin requirements, plasma triglyceride and cholesterol levels, and urinary glucose excretion. Improvements in glucose tolerance for normal individuals on diets of this type have also been noted.[5]

Trace-element malnutrition may contribute to the etiology or exacerbation of diabetes. Improved glucose tolerance and insulin sensitivity have been reported in NIDDs who have received glucose tolerance factor (GTF), a Cr(III)-complex containing nicotinic acid and amino acids (such as glycine, cysteine, and glutamic acid), or other Cr(III)-rich foods.[5,22] GTF has been plagued with problems of characterization, analysis, and stability.[23] Pharmacological testing of synthetic "GTF-like" compounds has afforded some success.[23] A symposium on the role of Cr in nutrition and metabolism was held in 1979.[24a] Dietary deficiency of Zn(II), a constituent of insulin, causes a reduction of insulin output.[5] A discussion of other trace elements, in addition to Cr, as nutritional factors in glucose homeostasis is available.[24b]

INSULIN

Biosynthesis and Recombinant DNA Techniques - Proinsulin is firmly established as the direct biosynthetic precursor of insulin. Also, the subcellular organization of the insulin biosynthetic mechanism of the β-cell is well understood.[25-28] The connecting (C) peptide, which induces the correct secondary and tertiary structure of proinsulin in the rough endoplasmic reticulum,[26,28] is excised in the Golgi apparatus to yield insulin. Work on chemical total syntheses of C-peptides and human proinsulin (1) has been described.[26,29-31]

Research on insulin messenger RNA (mRNA) has revealed the involvement of a larger polypeptide, preproinsulin, in insulin biosynthesis at the ribosomal level.[32-36] Complimentary DNA (cDNA) from reverse transcription of rat preproinsulin mRNA was first cloned in bacterial plasmids

by Ullrich et al.[37] A fused penicillinase-proinsulin translation protein was also generated in a bacterial clone.[38] Nucleotide sequence data gave the amino acid sequences of rat preproinsulins I and II.[37-39] The entire nucleotide sequence of human preproinsulin cDNA has been determined, confirming the amino acid sequence of human proinsulin (1) and suggesting the amino acid sequence of human prepeptide.[40,41] Radiolabeled, cloned human preproinsulin cDNA facilitated the isolation and structural characterization of the complete human insulin gene (two allelic variations),[42,43] which is located on chromosome 11.[44]

Genes for both insulin chains were designed (using the amino acid sequence for human insulin and the genetic code) and chemically synthesized with an extra methionine codon at the N-terminus of each insulin-gene sequence.[45] The "designer genes" were inserted into a bacterial gene coding for β-galactosidase, a secreted protein.[46] After expression in E. coli, the secreted, fused β-galactosidase-insulin A (or B) proteins were cleaved with BrCN at the site of the methionine residues to liberate the A or B chains; the two chains were joined by oxidation to give active insulin.[46] Biosynthetic human insulin is currently undergoing clinical testing[47] (as is semi-synthetic human insulin from chemical modification of porcine insulin by substitution of the sole distinguishing amino acid).[48] Two lucid reviews on the application of recombinant DNA technology to insulin synthesis are available.[49,50]

Since recombinant DNA methods allow the molecular manipulation of large proteins, by gene modification/expression, hormone analogs can be produced with amino acid substitutions, deletions, and/or additions. Expression of a chemically synthesized gene for an analog of human proinsulin in which the C-peptide is contracted from 35 to 6 amino acids has been achieved.[51]

Glucose stimulates proinsulin biosynthesis by enhancing the translational efficiency of proinsulin mRNA; translational control may ensure rapid gluco-regulation of proinsulin production.[52] Regulation of insulin biosynthesis by other agents has also been discussed.[53] A review on glucose control of insulin release and biosynthesis has appeared.[54]

Structure and Conformation - X-Ray crystallographic work on insulins has been reviewed.[55,56] X-Ray studies on porcine 2-zinc-insulin hexamers show, for each monomeric unit, a globular structure with a hydrophobic core. Circular dichroism studies suggest similar structures for porcine[57] and bovine[58] insulins in solution, under conditions of association; bovine insulin adopts a different conformation in dilute solutions.[58] Various NMR studies on insulins have been reported.[59]

Structure-Activity Relationships - An excellent review is available on numerous insulin analogs and derivatives.[60] Some recent papers on insulin analogs have appeared,[61-67] one of which describes the first analog, [21-asparaginamide-A]insulin, to display a divergence between receptor affinity and in vitro biological activity.[63] A novel semi-synthetic procedure for insulin derivatives,[68] involving trypsin-catalyzed peptide-bond formation at Arg^{B22}, permitted preparation of analogs with amino acid substitutions at the C-terminus of the B chain.[67] $[Leu^{B24}]$ insulin possesses insulin agonist/antagonist properties.[67]

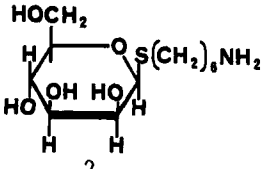
Receptors - Binding to specific receptor sites is an important aspect of insulin's action. Receptors on target-cell membranes appear to mediate short-term effects of insulin on glucose transport. The molecular events between binding and the biological response are not well understood.

Several reviews on insulin receptors cover: (1) insulin binding, (2) membrane-receptor properties and solubilization, (3) insulin degradation related to binding, (4) intracellular responses to binding, (5) insulin entry into cells, (6) intracellular receptors, (7) association with glucose transport, and (8) pathological relevance.[25,69-77]

The binding of insulin to receptors gives curvilinear Scatchard plots and non-competitive dissociation kinetics. Several proposals have been offered to explain this non-classical behavior.[78,79] Two insulin-binding components exist in detergent-solubilized material from fat- and liver-cell membranes;[79,80] also, two insulin receptor populations have been identified on human erythrocytes.[81] Various insulin receptors contain disulfide bonds that may connect two or more receptor subunits.[82,83] Adipocyte receptors possess at least two functionally distinct subclasses of disulfide bond, whereas liver receptors do not.[84] Data supporting the importance of receptor aggregation in insulin binding have been published.[85,86] Adipocyte receptors exist in an aggregated state prior to insulin binding,[84,87] but liver receptors may not.[84] A critical commentary on this area has appeared recently.[88]

Many research groups have begun to explore chemo- and photo-affinity labels for the insulin receptor to characterize and identify the receptor directly.[82,83,89-96] The functional insulin receptor in various rat tissues consists of, at least, a 130K-recognition and a 90K-effect/modulator subunit.[96]

Based on the involvement of carbohydrates in the function of the insulin receptor, many saccharide derivatives have been examined for insulin agonist and antagonist properties.[97-99] SARs for the glycosides indicate the importance of both sugar and aglycon moieties for activity.[97,99] The best *in vitro* insulin-like activity (stimulation of glucose oxidation in rat adipocytes) was found for β-D-mannopyranoside **2**, which also exhibited *in vivo* insulin-like effects.[97] The mechanism for the insulin-like properties of **2** and its congeners was attributed to generation of H_2O_2 during their metabolism by an amine oxidase, rather than to insulin receptor interaction.[97,99,100] However, the activity of antagonists of insulin-stimulated glucose oxidation in adipocytes was not associated with H_2O_2 production.[99] Several saccharide antagonists competitively inhibited insulin binding to insulin receptors.[99]



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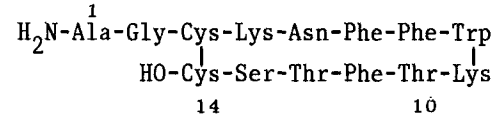

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Additional information on insulin's myriad mechanisms of action may be found in various review articles.[25,69,70,76,77,101-107]

Insulin-Delivery Devices - Insulin-injection therapy may not prevent the severe complications of diabetes.[6] Thus, much research has been directed toward achieving a closer imitation of the physiologic pattern of insulin secretion, mainly via pancreatic and islet-cell transplantation,[5,108-111] and artificial insulin-delivery systems.[5,109-113]

Portable pumps for continuous or preprogrammed, intravenous or subcutaneous insulin infusion (open-loop systems) have greatly improved blood-glucose control;[114-121] also, normal lipid and amino acid metabo-

lism have been restored in diabetics.[117] Long-term use of open-loop devices should establish whether regulation of the various metabolic defects attendant to diabetes will obviate the complications. Computerized glucose-sensing insulin-and-glucose-delivery devices ("artificial endocrine pancreas"; closed-loop systems), which closely approximate the state of glucose regulation found in nondiabetics, have been developed but not generally applied.[5,110-113,122] Devices based on living islet cells immobilized in hollow, semipermeable fibers are also under study.[5,110,123-125] A procedure involving microencapsulated islets, implanted into streptozocin-diabetic rats, has achieved marked success.[126] Islet-cell devices afford the advantage of supplying glucagon and somatostatin, as well as insulin.

A novel glucose-controlled insulin-delivery system based on a semisynthetic insulin bound to lectin has been described.[127] Insulin was covalently modified with maltose via glycosylation and Amadori rearrangement. The maltose-insulin adduct, having 78-95% of the bioactivity of unmodified insulin, was displaced by excess glucose from concanavalin A. Regulation by ambient glucose concentrations of an exogenous "insulin" may be achievable with such lectin-glycoinsulin complexes contained in hollow, semi-permeable fibers.

Liposomes[128] containing insulin offer an opportunity for development of an oral form of the hormone, but problems must be overcome.[129,130]

GLUCAGON

Glucagon, a 29-amino acid protein secreted by pancreatic α-cells in response to hypoglycemia (and/or amino acids), elevates blood-glucose levels by stimulating glycogenolysis and gluconeogenesis. This hormone may play an essential role in the pathogenesis of diabetes, along with insulin ("bihormonal abnormality hypothesis").[131-134] While this view has engendered much controversy,[132-136] selective glucagon inhibitors might offer a new approach to the management of diabetes.[132-135]

Reviews on glucagon covering structure, conformation, biosynthesis, synthesis and chemical alteration, SAR, receptors, and mechanisms of action have been published.[137-140]

Evidence for proglucagon, as a precursor to glucagon in pancreatic α-cells, has appeared.[141] A new 18,000-dalton protein was isolated, which readily converted to glucagon (3,500 daltons) and a new 10,000-dalton protein (a process analogous to the conversion of proinsulin to insulin). Glucagon biosynthesis was recently discussed.[142]

SOMATOSTATIN

Somatostatin (3), a potent tetradecapeptide hormone with multiple biological activities,[143-151] has been proposed as a potential agent for the treatment of diabetes, mainly because it suppresses release of glucagon.[143] However, 3 is rapidly degraded *in vivo* by cleavage, mainly at the Trp^8-Lys^9 peptide bond, and influences the secretion of other hormones (e.g., it inhibits insulin release). Work on somatostatin has been extensively reviewed.[138,143-151,169]

Biosynthesis - A putative prosomatostatin has been isolated from both porcine intestines[152] and hypothalami,[153] and identified as an octacosapeptide (somatostatin-28, SS-28) comprising the sequence of 3 with an

N-terminal extension of H_2N-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys. Ovine hypothalami yielded two bioactive somatostatin-like proteins: SS-28, which is structurally identical to the porcine compound, and SS-25, which has the structure of SS-28 minus the first three N-terminal amino acids.[154] Synthetic SS-28 was prepared by solid-phase methodology and found to be identical with the native porcine material.[155] On a molar basis, SS-28 showed equivalent inhibition of growth hormone release, two-fold greater suppression of glucagon, and ten-fold greater suppression of insulin, compared to 3.[155] A larger (4 x SS-28) protein from pancreatic islets has been identified as a prosomatostatin, which gave 3 on proteolysis and was associated only with the islet δ-cells.[156] Biosynthetic studies suggest that intracellular transport and processing of prosomatostatin are similar to that of other hormone precursors.[157]

Translation of mRNAs from anglerfish islets produced two preprosomatostatins, originating from separate genes.[158] Recombinant plasmids, containing cDNA related to mRNA that codes for preprosomatostatin, were cloned, and one of the cDNAs was sequenced.[159] Consequently, a sequence was deduced for a 119-amino acid protein comprised of 3 at the C-terminus (followed by a stop codon), a leader prepeptide of 18 hydrophobic amino acids, and a cryptic sequence of ca. 80 amino acids.[159]

A chemically synthesized gene for 3 was expressed in E. coli.[160] The methionine-β-galactosidase-linkage technique (see above) allowed release of active 3 from the composite polypeptide product.[160]

Analogs - For a potential treatment of diabetes, researchers have sought metabolically stable somatostatin analogs, which selectively inhibit the secretion of glucagon, as opposed to insulin (possibly still inhibiting growth hormone). [D-Cys^{14}]Somatostatin (4), as well as its Ala^2 (5) and D-Trp^8 (6) derivatives, inhibited arginine-induced glucagon release much more than insulin release; 4-6 were more potent than somatostatin.[161-163] [Ala^2-D-Trp^8-D-Cys^{14}]Somatostatin (7) suppressed arginine-induced glucagon, but not insulin, release.[164] While 7 potentiated glucose-induced insulin release, 4 and 6 did not.[164] Other workers have seriously questioned the glucagon selectivity of analogs 4 and 6.[165] Des-Ala^1,Gly^2 [His^{4,5}-D-Trp^8]somatostatin (Wy-41,747) exhibits prolonged activity as a selective inhibitor of glucagon and growth hormone release.[166-168] Several small-ring des-amino-acid analogs of 3 have shown selectivity between insulin, glucagon, and growth hormone secretion.[170]

Several articles discuss somatostatin analogs.[144,146,147,149,170] Recently, conformationally restricted bicyclic derivatives have been described, many of which are very active but have little selectivity between inhibition of glucagon and insulin.[171-173] Bicyclic analog Wy-40,391 inhibits growth hormone, but not insulin or glucagon release.[174] Long-acting analogs with specificity for inhibiting the release of growth hormone and growth hormone/insulin have been reported.[175,176]

Conformational aspects of 3 and its analogs have been studied and discussed.[171,177-180] A model for a biologically active conformation, based on bicyclic analogs and NMR studies, has been proposed.[171,177]

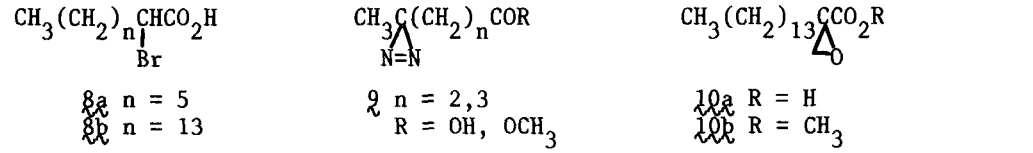
INHIBITORS OF FATTY ACID OXIDATION

Under conditions of high lipid mobilization, the products of free fatty acid (FFA) oxidation depress muscle-glucose utilization and maintain maximal rates of gluconeogenesis.[181,182] Randle and coworkers

proposed that glucose and FFA control each other's release and utilization under physiological conditions ("glucose-fatty acid cycle") and suggested that excessive FFA oxidation may be a key factor underlying decreased glucose tolerance in diabetes. Thus, correction of abnormally high rates of FFA oxidation may be useful for treatment of diabetics. This view gained support when hypoglycin, 4-pentenoic acid, (+)-decanoylcarnitine, and α-bromo fatty acids, all of which inhibit FFA oxidation, produced hypoglycemia in animals.[183-185] However, these inhibitors are toxic, inhibit enzymes unrelated to hypoglycemia, and/or are not orally effective.[183-185]

Inhibition of FFA oxidation can be affected by various mechanisms. Recent studies[186,187] have indicated that a metabolite of 4-pentenoic acid inhibits 3-ketoacyl-CoA thiolase, one of the terminal enzymatic steps in mitochondrial β-oxidation, thus contradicting an explanation[188] that the inhibitory effect of pentenoic acid on FFA oxidation is due to its sequestration of CoA and/or carnitine as esters. 2-Bromooctanoic acid (8a) was recently reported to inhibit β-oxidation of FFA in perfused rat liver and in rat-liver mitochondria by a mechanism different from that of 2-bromopalmitic acid (8b).[189] Treatment of rats with 8b lowers blood glucose[190] and enhances the ability of hemidiaphragms to oxidize glucose, secondary to inhibition of FFA oxidation. The hypoglycemic activity of various diazirines 9 was suggested to occur via inhibition of hepatic fatty acid oxidation.[191]

Interest in inhibition of fatty acid oxidation has been sparked by two new discoveries. (1) The activation of hepatic FFA oxidation and ketogenesis associated with fasting or diabetes were found to depend on the decline of an endogenous inhibitor of FFA oxidation, malonyl-CoA.[192,193] Malonyl-CoA, which is a competitive inhibitor of carnitine palmitoyl transferase I (the first enzyme required for the oxidation of long-chain FFA), is the first unique intermediate of the opposing lipogenic pathway.[194] (2) The long-chain fatty acid derivative, 2-tetradecylglycidic acid (McN-3802, 10a), as well as its methyl ester 10b (McN-3716), were not only found to be potent, irreversible inhibitors of carnitine palmitoyl transferase I *in vitro*[185,195] and *in vivo*,[185,196] but also were found to exhibit promising pharmacologic activity. Both 10a and 10b, which are more potent than other inhibitors of FFA oxidation, produce oral hypoglycemic activity in normal and diabetic rats, depancreatized dogs and db/db mice,[199,200] and reverse ketoacidosis in diabetic rats and dogs.[199] Glycidates 10a and 10b lower glucose concentrations only under conditions where FFA are used as major energy substrates (fasting, diabetes, high-fat diets), not under conditions where energy is derived from carbohydrate. Many related fatty acids, including glycidate, epoxide, and α-substituted analogs, do not produce the effects of 10a and 10b.[197-199,201,202] Studies using rat hepatocytes[200] and hemidiaphragms[197] indicated that the hypoglycemic effects of 10a and 10b result from increased utilization of peripheral glucose and decreased gluconeogenesis, because of their inhibitory effects on the oxidation of long-chain (but not short-chain) FFA.



8a n = 5
8b n = 13

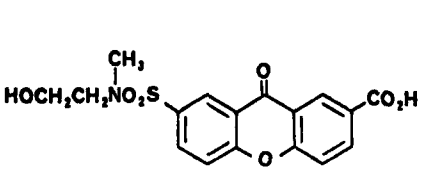
9 n = 2,3
R = OH, OCH_3

10a R = H
10b R = CH_3

ALDOSE REDUCTASE INHIBITORS

Studies in animal models and man suggest that conversion of glucose to sorbitol via the polyol pathway may be important in the pathogenesis of diabetic cataracts, retinopathy, and peripheral neuropathy.[203-205] Elevated levels of sorbitol, detected in lens[204] and peripheral nerves[205] of diabetics, are thought to arise from the action of the NADPH-linked enzyme aldose reductase (AR), which catalyzes the conversion of sugar aldehydes to their respective sugar alcohols. A description of this enzyme and evidence for its role in chronic complications of diabetes were published in 1978.[6]

Several new drugs which inhibit AR have been reported. Chromone carboxylic acids,[206] which exhibit inhibitory activity in vitro, led to the discovery of a series of 7-substituted xanthone-2-carboxylic acids,[207] the most potent of which, 11, produces an 83% inhibition in vitro at 1 μM. Activity is retained when the $-SO_2NMe-$ is exchanged for -S-, -SO- and $-SO_2-$, but methylation of the free alcohol function results in greatly diminished activity. Sorbinil (CP-45,634, 12), a potent, non-competitive inhibitor (IC_{50} ~0.5 μM) of AR was orally effective in decreasing polyol accumulation in lens and sciatic nerve of galactosemic and streptozocin-diabetic rats.[208] While doses as low as 0.25 mg/kg were effective in inhibiting sorbitol accumulation in diabetic rats, doses up to



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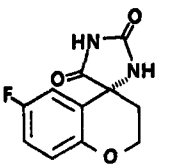

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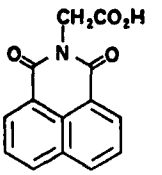

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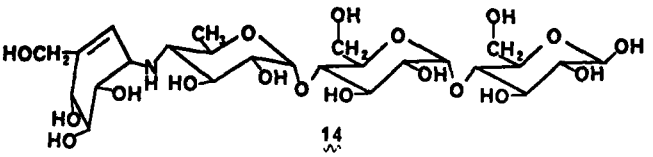
20 mg/kg did not alter fasting blood glucose or glucose tolerance. Alrestatin (AY-22,284A, 13), an inhibitor of AR in animals, did not ameliorate diabetic neuropathy in man as reflected by measurements of sensory- and motor-nerve conduction velocities.[209] Studies of AR inhibitors have been hampered by the lack of an in vivo noninvasive method for directly measuring enzyme inhibition.

METHODS OF SLOWING CARBOHYDRATE ABSORPTION

Recent studies have indicated that reduction of postprandial fluctuations in blood glucose and insulin occur when diabetics consume meals containing unabsorbable carbohydrates, such as guar gum and pectin.[210-212] Guar, a gum produced from the seeds of the Indian cluster bean (Cyamopsis tetramaloba), may exert its effect by forming intraluminal gels in the small intestine, thus causing a delay in glucose absorption.[213]

Carbohydrate absorption may be impaired by inhibiting enzymes involved in the breakdown of starch and sucrose. Some success was obtained in early studies[214,215] using the protein α-amylase inhibitors, BAY-e-4609 and BAY-d-7791, but these agents did not prove to be of therapeutic value in either diabetics or obese patients.[216] However, a new competitive α-glucosidase inhibitor, a complex oligosaccharide of microbial origin, acarbose (BAY-g-5421, 14), was effective in delaying starch and sucrose digestion in the small intestine.[217,218] Acarbose reduced the

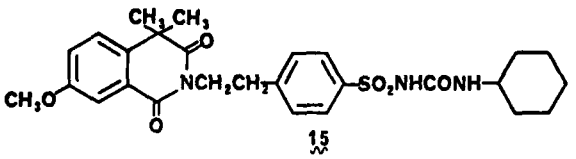
incremental increases of blood-glucose and serum-insulin in starch- and sugar-loading tests with animals and humans.[216,218,220] It also lowered and stabilized blood-glucose levels in diabetics when given with meals, and provided adjunctive benefit to patients on sulfonylurea and insulin therapy by lowering and smoothing blood-glucose profiles.[221-223] Decreases of body weight and lowering of triglyceride and cholesterol levels have been reported in animals,[217,225] but not in man.[223,224] The use of acarbose has been limited by side effects, including flatulence and diarrhea resulting from colonic fermentation of malabsorbed sugars.[221-224] Some investigators successfully used low doses of acarbose along with guar to minimize these undesirable effects.[226]



14

ORAL HYPOGLYCEMIC AGENTS

Sulfonylureas, Sulfonamides, and Carboxylic Acids - When given acutely, sulfonylureas lower blood-glucose levels by stimulating insulin secretion from β-cells. However, chronic administration of these agents to NIDDs, or laboratory animals, reduces insulin secretion and proinsulin biosynthesis.[227] Thus, the long-term efficacy of sulfonylureas cannot be explained on the basis of pancreatic effects alone. Recent research has identified extrapancreatic effects of sulfonylureas, the most promising of which is an ability to increase the number of insulin receptors. Both gliquidone (ARDF-26, 15)[228] and glipizide[227] increase the number of insulin receptors in different mouse models. Glyburide (glibenclamide) inhibits insulin-induced receptor loss (down regulation) in isolated human fibroblasts.[229] Monocytes from obese NIDDs, under treatment with both glyburide and diet, exhibited augmented insulin-binding affinity and increased numbers of insulin receptors, compared to monocytes of NIDDs on dietary therapy alone. These changes may explain the enhanced insulin sensitivity in patients taking glibenclamide.[230]

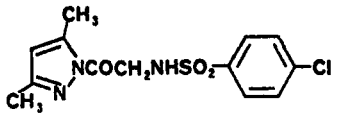


15

Although long-term efficacy of sulfonylureas has remained a point of controversy, certain investigators believe that proper diet and patient selection are necessary for successful long-term management of NIDDs with these agents.[2,231] A recent ten-year study of glucose-impaired patients concluded that diet-plus-tolbutamide therapy may postpone or prevent the onset of NIDD.[232] Adverse effects of the sulfonylureas were reviewed recently.[233]

A novel sulfonamide, AD-58 (16), causes dose-dependent lowering of blood-glucose and FFA levels in alloxan-diabetic rats.[234] Meglitinide (HB-699, 17) reduces blood-glucose concentrations in rats and dogs, and causes a biphasic release of insulin in unfasted dogs.[235] The relative

hypoglycemic potencies of HB-699, the second-generation sulfonylureas, and the sulfonamides have been rationalized on the basis of an adjacent two-site "sulfonamide-receptor" model.[236]



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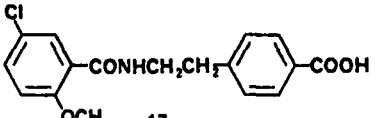
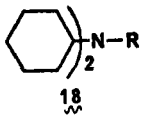


17

Dichloroacetate - The mechanism by which dichloroacetate (DCA) reduces fasting hyperglycemia and hyperlipoproteinemia in diabetic patients has been a subject of intense interest.[237] DCA activates pyruvate dehydrogenase by inhibiting pyruvate dehydrogenase kinase, thus reducing the availability of alanine, lactate, and pyruvate, which are precursors for hepatic gluconeogenesis. Direct effects of DCA on gluconeogenesis in isolated rat hepatocytes and on lipogenesis in rat kidney have been reported.[238-242] However, studies with DCA in fasted dogs failed to demonstrate alterations of blood-glucose concentration and rate of glucose production, despite substantial decreases in gluconeogenesis-precursor levels.[243] Thus, DCA may produce hypoglycemia by direct stimulation of glucose utilization.[243] DCA shows promise for reversing biguanide-induced lactic acidosis in man.[244,245]

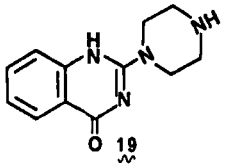
Amines, Amidines, Guanidines, and Related Compounds - Dicyclohexylamines 18a and 18b decrease blood-glucose levels in fasted mice and streptozocin-diabetic rats,[246] presumably via stimulation of glucose metabolism in muscle tissue.[247]



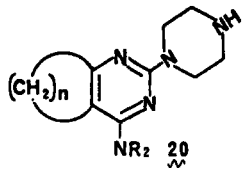
18

a R = H

b R = Et



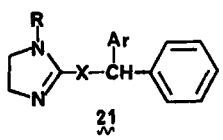
19



20

a n = 3 R_2 = H_2

b n = 4 R_2 =

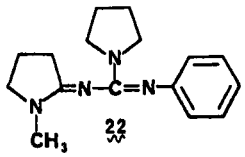


21

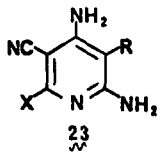
R X Ar

a Me NH Ph

b H CH_2 2-Py



22



23

a R = H X = Br

b R = H X = I

c R = CN X = Br

Centpiperalone (68/157, 19) is reportedly undergoing Phase I clinical trials.[248] Diaminocycloalkyl-fused pyrimidines 20a and 20b, modeled after centpiperalone, showed marked hypoglycemic activity in mice pretreated with 2-deoxy-D-glucose.[249] Two research groups independently discovered the hypoglycemic activity of benzhydrylaminoimidazoline 21a

(McN-3734),[250-252] a compound related to an earlier series of amidines.[253] Of a large number of analogs involving benzhydryl and benzhydryl-like groups in combination with amidine and guanidine functionalities, 21b demonstrated the greatest hypoglycemic activity and safety.[254]

A novel series of potent oral hypoglycemic agents was reported in 1977.[255,256] Pirogliride (McN-3495, 22), selected for detailed study, displayed potent oral and parenteral activity in rats, dogs, mice and Rhesus monkeys; in monkeys a ten-fold greater potency was observed.[257] Not only does 22 differ in structure from sulfonylureas and biguanides, but also in mechanism of action.[257,258] Pirogliride showed an insulin-secretagogue effect, without altering glucagon secretion, in vivo and in the perfused rat pancreas.[259] Studies have demonstrated that 22 enhances insulin secretion in the presence, but not in the absence, of glucose, whereas tolbutamide only weakly enhances insulin secretion in the absence of glucose.[259,260] Also, 22, unlike tolbutamide, directly stimulates glucose metabolism and partially reverses the inhibition of glucose-stimulated insulin secretion by mannoheptulose.[260] A nonpancreatic mechanism is also likely because 22 stimulates glucose oxidation in muscle tissues[261] and shows hypoglycemic activity in genetically diabetic (db/db) mice and streptozocin-diabetic rats.[257]

Three 2,4-diamino-6-halopyridine derivatives, containing one or two cyano groups (23a-23c), caused a three- to four-fold potentiation of glucose-stimulated insulin secretion at 100 μM in the perfused rat pancreas.[262,263] However, 23a (50 mg/kg, p.o.) did not lower blood-glucose concentrations in animals subjected to an i.v. glucose-tolerance test, even though insulin levels were increased three-fold.[262]

Effects of certain guanidines and biguanides on mitochondrial membranes and respiration have been reviewed.[264]

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Chapter 18. New Agents for the Treatment of Inflammation and Arthritis

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Introduction — Non-steroidal antiinflammatory (NSAI) agents are used mainly to control the symptoms of arthritis and related connective tissue diseases. Immunoregulatory agents are increasingly being investigated for their effect on immunologically-mediated forms of arthritis, such as rheumatoid arthritis (RA). New compounds in both these classes continue to be discovered in animal models of inflammation. In addition, recent clinical reports have established a number of compounds from both classes as being effective in treating inflammatory diseases. This review will survey the pharmacological, medicinal and clinical literature from late 1977, when this topic was last reviewed,[1] through all of 1980. The more potent new NSAI agents, as well as the immunoregulatory agents of potential use in arthritis, will be discussed in this review. Special attention will be given to compounds reported to be potent in animals or man by the oral route, since high doses by other routes are known to produce false positive results due to toxicity or local irritating effects.

There are many reviews of the literature on the well-established NSAI agents.[2-8] Side effects of the NSAI agents have been discussed,[9,10] and a number of NSAI compounds have been compared in standard animal models.[11,12] The more general topics of inflammation [13-15] and the control of inflammation[16] have been reviewed. An in-depth survey of antiinflammatory drugs has been published,[17] while the proceedings of several symposia dealing with NSAI drugs[18-22] and reviews of the literature on arthritis[23-25] and the immunopathology of RA[26,27] have appeared.

Mechanisms of Action — Defining the principle modes of action of NSAI drugs has been hampered by the many pharmacological effects observed with these agents. Modern reviews of this subject provide useful overviews of the diverse activities viewed by some as relevant to the clinical activity of these drugs.[28-31] Four areas are currently receiving particular attention: the effect of NSAI's on: a) arachidonic acid metabolism; b) highly active oxygen species; c) inflammatory cell movement; and d) the preferential distribution of acidic NSAI's into inflammed tissues.

Products derived from arachidonic acid (AA) play diverse roles in inflammation. In contrast to steroids, which are found to prevent AA release by inhibiting phospholipase A_2 activity,[32] NSAI agents typically inhibit cyclooxygenation of arachidonic acid[33,34] and, thereby, inhibit prostaglandin formation. The finding that lipoxygenase pathway products, such as 5,12-HETE (leukotriene B), are very potent chemotactic substances[35,36] found in RA synovial fluid,[37] has stimulated research toward finding agents to block this particular pathway of AA metabolism. Prostaglandins from chondrocytes may be involved in mediating cartilage destruction in arthritis,[38] suggesting a more fundamental role for AA metabolites in the pathology associated with arthritic disease.

Highly active oxygen species, such as superoxide anion and hydroxyl radicals, derived from activated phagocytes, may contribute to the pathogenesis of arthritic disease.[39,40] Potent chemotactic factors generated by superoxide[41,42] are suppressed by treatment *in vitro* with standard NSAI drugs[43] and may partly explain their mechanism of action. Hydroxyl radical formation is also suppressed by NSAI's[44,45] and by dimethylsulfoxide.[46]

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The ability of phagocytes to secrete chemotactic factors at an inflammed site is well established.[47-49] A variety of *in vitro* and *in vivo* models have demonstrated the ability of NSAI's to suppress cell migration into an inflammed site, suggesting this property as a possible mechanism of action.[50-52] However, clinical support for this hypothesis is still lacking.

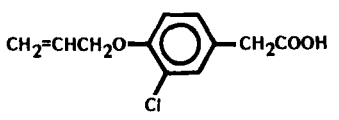
The preferential distribution of acidic NSAI agents into inflammed sites, as well as into stomach lining, liver and kidneys, contributes to their activity and may help explain their side effect profile.[53,54]

NSAI's bind to plasma proteins; some, but not all, can displace plasma-bound corticoids in rats.[55] This property may contribute to the antiinflammatory activity of some NSAI's.

Compounds Under Investigation

Immunoregulatory Agents — With so many NSAI compounds available to treat the symptoms of arthritis, there has been an intensive effort in many laboratories to find immunoregulatory agents (sometimes termed "disease-modifying" or "antirheumatic" agents) to halt the arthritic process. Agents are needed with greatly improved toleration over gold, chloroquine, penicillamine and the immunosuppressive agents. Reviews of the literature on the older disease-modifying agents are available.[56-61] This review will be restricted mainly to low molecular weight compounds reported to be active in animal models of potential relevance to RA.

In the course of routine testing in RA, a few apparently classical NSAI drugs have been reported in single clinical trials to have "an effect on the disease process," but these findings remain to be confirmed. Alclofenac (**1**),[62] for example, is such a compound; more recent double-blind clinical results,[63] however, have failed to demonstrate any immunological effects with this compound. Two other typical NSAI acetic acids, fenclofenac[64,65] (**2**) and clozic[66,67] (ICI 55897, **3**), are claimed to do more than control inflammatory symptoms, but these claims, which are based on limited data, must await confirmation in large, controlled clinical trials. Similarly, the non-acidic compound proquazone[68] (**4**) has been found in one study to influence immunoglobin levels in arthritic patients with some inhibition of bony erosions.[69] This finding also requires confirmation.



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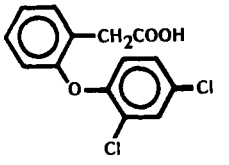

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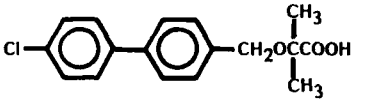

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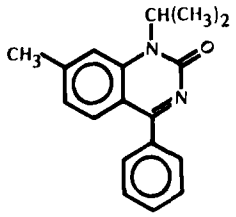
Of the immunoregulatory agents observed to restore immune parameters to normal, levamisole (LVS, **5**) is clearly the most thoroughly studied in RA. The literature on this compound has been repeatedly reviewed,[70-72] and the proceedings of a symposium have been published.[73] Although LVS is now generally accepted as being effective in RA over a background of NSAI therapy, adverse side effects are often severe and lead to high withdrawal rates on long-term use.[74-76] Restoration of certain immunological parameters, such as E-rosettes[77] and PHA response[78] in RA patients treated with LVS, has been reported to correlate with clinical improvement. A hydrolysis product of LVS, the thiol OMPI (**6**)[79], has been found to be more potent than LVS itself in stimulating lymphocyte proliferation[80] and carbon clearance in mice,[81] suggesting that **6** may be an active metabolite *in vivo*.



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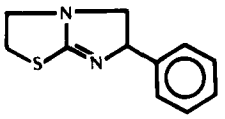

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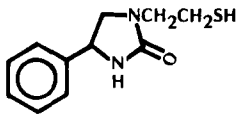

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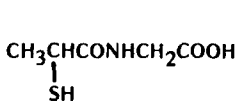
Other immunoregulatory agents with improved toleration are being actively sought. Preliminary reports and abstracts have appeared on a number of such compounds. Two thiols, thiopronine (**7**) and SA-96 (**8**), appear to be structurally related to penicillamine. Thiopronine (**7**)[82] has been examined in RA patients, but only 6 of 22 patients responded, while 9 were discontinued due to side effects.[83] SA-96 (**8**) exhibits curative effects in rat adjuvant arthritis[84] and produces increased plaque-forming cells in mice.[85] Pyrithioxine (**9**)[86] has been examined in an open RA trial with 54% of patients showing beneficial effects.[87] Double blind trials will be needed to determine the true effect and the frequency of side effects with this agent.



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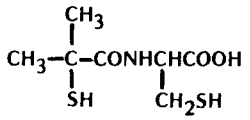

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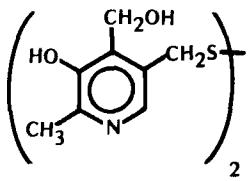

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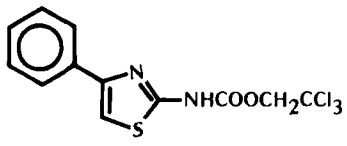
More diverse types of structures are represented by F-1686 (**10**), NPT-15392 (**11**) and therafectin (SM-1213, **12**). F-1686 stimulates lymphocyte responses while decreasing leukocyte counts in a hypersensitivity skin reaction in guinea pigs.[88] NPT-15392 (**11**)[89] has been shown to stimulate human lymphocytes[90] and to increase active rosetting cells *in vitro*.[91] Therafectin (**12**) inhibits synovitis in rabbits[92] and reduces weight loss in virus-



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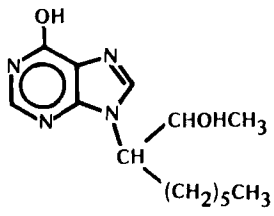

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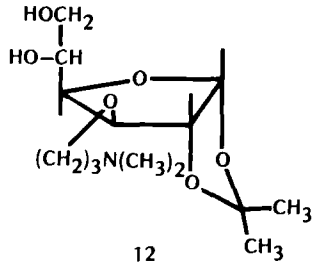

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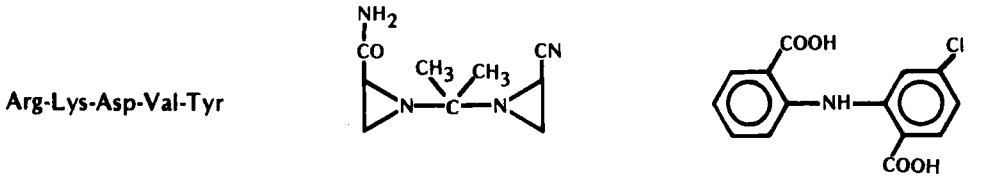
challenged hamsters.[93] Thymopoietin pentapeptide (TP-5, **13**) restores low rosetting cells of RA patients to normal levels after intravenous dosing.[94] Azimexon (BM-12531, **14**)[95] has a similar stimulatory effect on the low rosetting cells of cancer patients[96] and is also capable of reducing the lethality of infectious agents administered to mice.[97] The anthranilic acid derivative CCA (**15**) enhances antibody responses,[98] inhibits adjuvant arthritis in rodents[99] and increases survival time in NZB mice.[100] The long-term toleration and efficacy in RA patients of all these agents remain to be determined.



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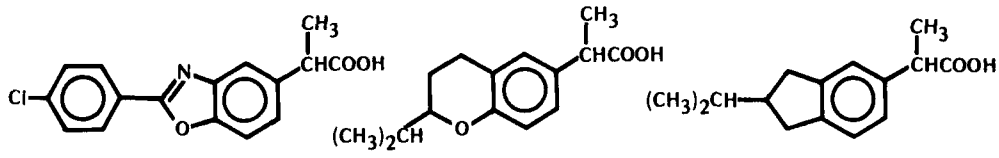
Cytotoxic immunosuppressive drugs have been reviewed,[101,102] but no major new agents have been reported. Frentizole, an immunosuppressive agent discussed in an earlier review,[1] was found marginally effective in systemic lupus, but serious toxicity caused discontinuance of clinical trials with this compound.[103]

NSAI Agents — These antiinflammatory compounds will be grouped into six categories: the arylpropionic acids, the acetic acids, miscellaneous carboxylic acids, enolic acids, prodrugs of various kinds that are converted *in vivo* to carboxylic acids, and the non-acidic antiinflammatory agents.

A. Arylpropionic Acids — Following the success of propionic acid derivatives such as ibuprofen and naproxen, the list of antiinflammatory propionic acids ("profens") continues to grow.

Benoxaprofen (**16**) is a moderately active antiiflammatory agent with relatively weak prostaglandin inhibitory activity and a long half-life in animals and man.[104,105] The effect of **16** on leukocyte migration is shared by several other NSAI's.[106] FPL-58302 (**17**)[107,108] and UP 517-03 (**18**)[109] are reasonably potent in the rat foot edema (RFE) model and have improved toleration in rats.



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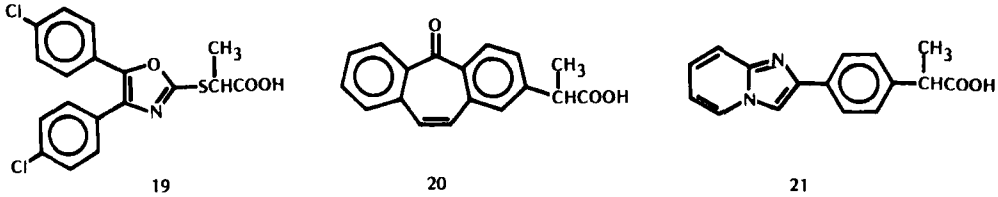
Tioxaprofen (EMD-26644, **19**) is an arylthiopropionic acid that inhibits rat adjuvant arthritis and platelet aggregation with a long half life.[110] The d-isomer of a dibenztropone propionic acid (**20**), structurally related to isoxepac (see below), is three times more potent than indomethacin in the rat adjuvant arthritis model.[111] Y-9213 (**21**) has an ED_{50} of 9 mg/kg in the RFE model[112] and inhibits lysosomal enzyme release *in vitro*.[113]



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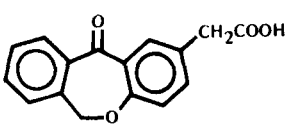
B. Arylacetic Acids — Isoxepac (HP 549, **22**) is a potent antiinflammatory arylacetic acid, with an ED_{50} of 6 mg/kg in the RFE model and gastric effects less than those of indomethacin

in rats.[114] A biphasic half-life for **22** in man has been observed.[115] A closely related arylacetic acid, oxepinac (**23**),[116] has potent RFE activity (ED_{50} 3.7 mg/kg)[117] and a half-life in man of about 1 hour.[116] Tiopinac (RS 40974, **24**), the sulfur isostere of compound **23**, is relatively well tolerated in animals[118] but is toxic in man[119] and no longer in clinical trials.



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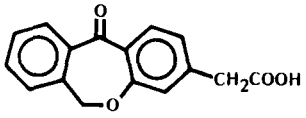

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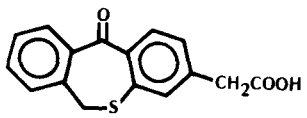

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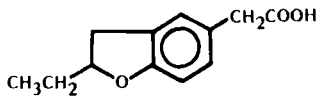
Furofenac (SAS-650, **25**) has been reviewed[120] and its metabolic transformations determined in various species.[121] Zidometacin (**26**)[122] is a direct analog of indomethacin where an azido function replaces the chloro substituent. The compound is quite potent (ED_{50} 4.7 mg/kg) in the RFE and less toxic in mice than indomethacin.[123] The cyclopenta[b]pyridine carboxylic acid (**27**), which may be considered as an arylacetic acid, has potent antiinflammatory activity and prostaglandin biosynthesis inhibitory activity.[124]



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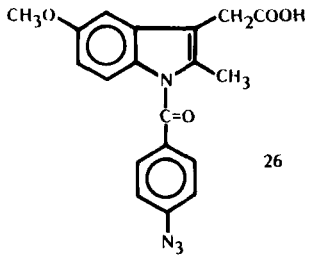

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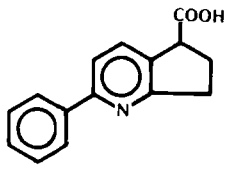

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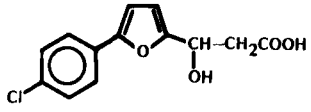
C. Miscellaneous Carboxylic Acids — Several other types of carboxylic acids have antiinflammatory activity. Orpanoxin (F-776, **28**)[125] is moderately active in the RFE (ED_{50} 61 mg/kg) and non-ulcerogenic in an acute test in rats.[126] Butibufen (**29**) is only weakly active in the RFE (ED_{50} 111 mg/kg),[127] but efficacy in RA trials has been observed.[128] The extensive literature on fenbufen (**30**) has been reviewed.[129,130] Evidence has been obtained for metabolic conversion of **30** to 4-biphenylacetic acid.[131]



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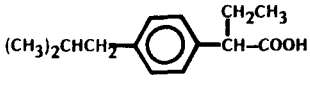

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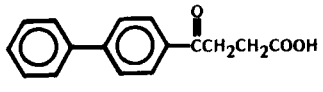

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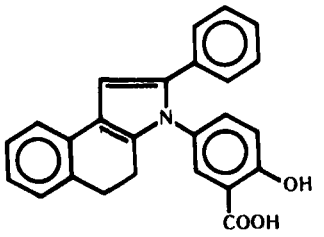
Fendosal (HP 129, **31**) is a salicylate derivative of similar potency to aspirin in animal models;[132] it exhibits analgesic activity in man.[133] A single report of an open trial in RA has appeared for diacetylrhein (**32**), a compound devoid of prostaglandin inhibitory activity, indicating some beneficial effects of the agent with no adverse effects on the gastric mucosa.[134] The tetrahydroindazole-5-carboxylic acid (**33**) is potent in the RFE (ED_{50} 3.5 mg/kg) test but causes some ulcerogenicity in rats.[135]



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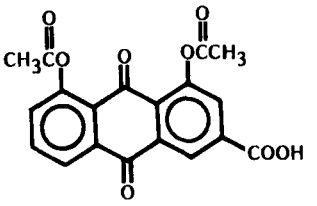

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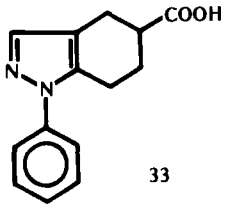

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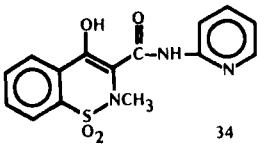
D. Enolic Acids — The literature on antiinflammatory 4-hydroxy-1,2-benzothiazine-3-carboxamides ("oxicams"), exemplified by the very potent agent piroxicam (CP-16,171, **34**), is expanding rapidly.[136] Extensive pharmacological and clinical publications on piroxicam[137-143] establish this compound as an effective, long-acting agent for the treatment of various inflammatory conditions in man. Metabolites of piroxicam are much less active than piroxicam as anti-inflammatory agents.[142]



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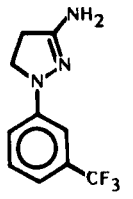
E. Prodrugs of Carboxylic Acids — Many prodrugs of the standard NSAI acids have been made by derivatizing the carboxylic function. Due to space limitations, only the names and key references will be given for some of these prodrugs. Prodrugs of aspirin include eterylate,[144] salsalate[145] and Abbott 45474.[146] Prodrugs of indomethacin include protacine,[147] oxamethacin[148] and acemetacin.[149] Prodrugs of ibuprofen include AF-2259.[150] Enolate prodrugs of the pyrazolidinediones have also been prepared (suxibuzone[151] from phenylbutazone and ITA-194[152] from prenazone). Although there are limited data in animals and man suggesting decreased gastric irritation resulting from the use of these prodrugs, no large, double-blind clinical trials in arthritis have been reported to support these claims.

F. Non-acidic Compounds — Several new non-acidic antiinflammatory compounds have been reported recently. BW-755c (**35**) inhibits both cyclooxygenase (CO) and lipoxygenase (LO) enzymes.[153] Compound **35** also inhibits leukocyte migration into the site of inflammation in rats,[154,155] although the classical NSAI agents also suppress leukocyte migration in this model.[155] Timegadine (SR-1368, **36**) is also reported to inhibit both CO and LO[156] and is fairly potent (ED_{50} 30 mg/kg) in the RFE.[157] Cloximate (DU 22599, **37**) was found in a small clinical trial to be effective for treating arthritis at a dose of 100 mg/day.[158] Chinoin-127 (**38**)



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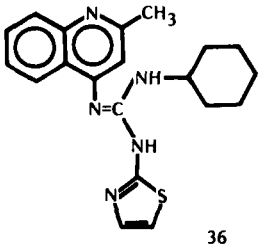

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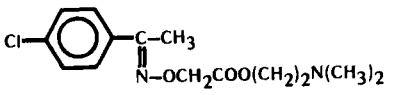

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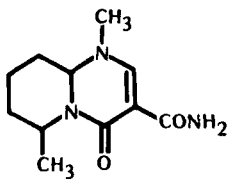
is moderately potent in the RFE and is reported to be in clinical trials.[159] Fluproquazone (**39**) has been shown to be an effective analgesic in post-operative pain.[160] Broperamole (TR-2378, **40**) has an ED_{50} in the RFE of 25 mg/kg and is effective topically in animal models.[161]



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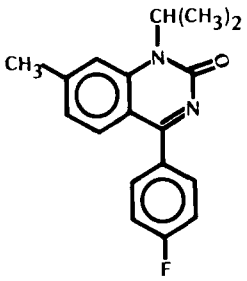

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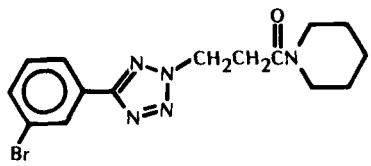

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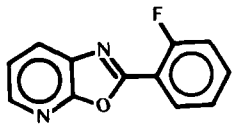
A family of oxazolopyridines, e.g. **41**, are potent inhibitors in the RFE model, exhibiting low gastric irritating properties.[162] Some triazine derivatives, e.g. **42**, were found active in both RFE and rat adjuvant arthritis.[163] Auranofin (**43**),[164,165] a novel gold salt, has exhibited beneficial effects in RA.[166] Continuing clinical trials seek to establish clear safety advantages over the older forms of chrysotherapy.



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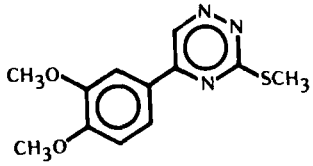

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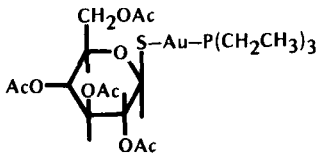

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Conclusion — Despite the many NSAI agents already available for treating arthritis and inflammation, newer drugs continue to appear. The new drugs are often used to treat patients not adequately controlled by existing agents. Some of the new agents require much lower daily doses than existing drugs to achieve effective control of inflammation. A very few of the new NSAI agents offer the additional advantage of a single daily dose. Decreased frequency of dosing can be expected to lead to improved patient compliance[167] and provide more uniform control of pain and inflammation.

Immunoregulatory agents for controlling the RA disease process are at a much earlier stage of clinical evaluation. Clinical trials in RA now support the efficacy of one immunoregulatory agent (levamisole),although the mechanism of action of this compound is not yet understood. Clearly, better toleration on chronic administration remains a desirable goal for new agents of this type.

In the absence of sufficient information concerning the causative agent in RA, and considering the common pathways of tissue destruction seen in RA and osteoarthritis, future research efforts are expected to be targeted at the processes which lead to tissue destruction. With the NSAI's available to control pain and inflammation, drug therapy for arthritis requires agents which will arrest the progressive joint destruction of this disease.

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Chapter 19. Peptides of the Hypothalamus

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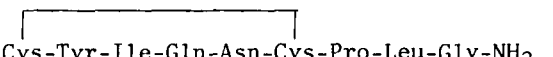
Introduction - This report summarizes major recent developments in the synthesis, structure-activity relationships and potential clinical applications of hypothalamic peptide hormones and their analogs. Other recent Annual Report chapters covering these areas included "Somatostatin,"[1] "Chemical Control of Fertility,"[2] "Peptide Conformation and Biological Activity,"[3] and two reports on "Peptide Hormones."[4,5] Interested readers are also referred to general reviews on hypothalamic peptides[6-8] (including specific discussions of LH-RH, somatostatin, and TRH) and the neurohypophyseal hormones.[9,10] Summaries of recent advances in peptide backbone modifications, conformational studies on peptides, and the increasing evidence of their role as neurotransmitters are briefly considered in separate sections of this report.

Neurohypophyseal Hormones

The neurohypophyseal hormones are synthesized in the hypothalamus and transported to the neurohypophysis where they are stored pending release upon appropriate stimulation. In man these hormones include oxytocin (1) and arginine vasopressin ([Phe^3,Arg^8]oxytocin). At least seven other closely related nonapeptides are found in various vertebrate species, and arginine vasotocin ([Arg^8]oxytocin) has been suggested as a common evolutionary precursor.[11] It has proven possible through suitable



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analogs to largely dissociate many of the biological actions of these peptides, including the memory consolidation properties of vasopressin analogs.[36] It has been hypothesized that oxytocin and vasopressin are synthesized ribosomally as prohormones, together with their appropriate neurophysin carrier proteins, in the hypothalamic paraventricular and supraoptic nuclei, respectively.[12] This hypothesis has now been supported by in vitro synthesis of these putative precursors.[13]

The molecular and cellular aspects of the neurophysins and the nature of neurophysin-hormone interactions have been reviewed, and the nature and consequences of oxytocin/vasopressin binding to their ultimate receptors is beginning to be deciphered.[14] A series of studies in this latter area, using about 15 hormone analogs, showed good correlation between analog binding and activity.[15]

Oxytocin - The activity enhancing properties of such general modifications as 4-threonine substitutions,[16] removal of the terminal amino group,[17] and replacement of the disulfide bridge by carba analogs,[18] have

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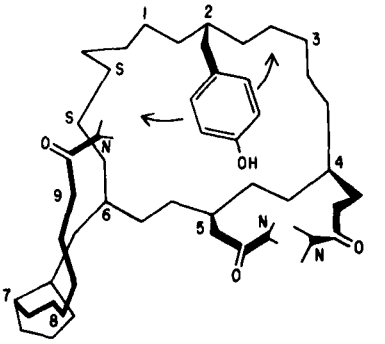
been well established and either retard biodegradation or increase the analogs' affinities to the receptor. Some of these modifications have been incorporated within potential hormone antagonists and contribute comparable potency enhancements. For example, greater antagonist potency (pA_2 = 7.55) was observed for [Pen^1,Thr^4] oxytocin relative to [Pen^2] oxytocin (pA_2 = 6.86).[19] The synthesis of a [Trp^8] oxytocin analog, designed as a fluorescence probe for an energy transfer experiment, gave an analog with both agonist and antagonist properties and prolonged duration of action.[20]

Recent progress in the design of antagonists to both oxytocin and vasopressin has demonstrated the applicability of cross-over information from the two systems, and further insights from agonist models, such as the highly active (and selective) [1-deamino, 2-phenylalanine, 7-(3,4-dehydroproline)]arginine vasopressin (with an ADH activity of 13,000 ± 1250 IU/mg) can be anticipated.[21] Such modifications as alkylated cysteine derivatives (penicillamine, etc.),[22] 2-O-alkyltyrosine, and halogenated tyrosines have proven additive with other new antagonist-inducing modifications. It was also recently shown that N-terminal acetylation gives impressive potency increases for antagonists of ADH, and further applications can be anticipated.[23]

New analogs of oxytocin designed to test varying aspects of the Urry-Walter and "cooperative" models[24] have been synthesized. In general, N-methylated analogs which prevent presumed intramolecular H-bonding interactions have nevertheless retained reasonable activity.[25] A C-terminal dimethyl amide derivative of oxytocin had only 3% of oxytocin's uterotonic potency, the bulkiness presumably interfering in attaining the correct receptor-active conformation.[26] A [D-Tyr^2] oxytocin derivative had reduced activity (8.4 ± 0.3 units vs. 546 ± 18 for oxytocin) in the rat uterotonic assay, also consistent with a proposed biologically active conformer (2) which requires the tyrosine-2 side chain folded over the ring and oriented closely to the asparagine-5 side chain, the other active element. An analogous [D-Tyr^2] arginine vasopressin analog retained nearly 50% of its parent's ADH activity, suggesting a similar orientation is not essential for binding to the antidiuretic receptor.[27] The selective rigidity of antagonists of oxytocin containing penicillamine-1 and related substituents compared to more flexible agonists has been cited as evidence in favor of a "dynamic model" for hormone action.[28] This model requires a hormone (and receptor) sufficiently flexible to permit a second transduction step following the initial binding interaction. Indirect support for such a view comes from evidence that hormone binding alters the conformation of the insulin receptor as monitored by differences observed in rates of trypsin proteolysis.[29] Several reviews have appeared covering these models.[10,30]



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Vasopressin - A more detailed model of the vasopressin structure has led to considerable improvements of potencies in both agonist and antagonist series.[31] The nature of vasopressin-neurophysin interactions and the identification of a glycopeptide AVP-NPII precursor[13,32] is complicated

by the isolation of two different undecapeptide derivatives from fresh frozen bovine neurohypophysis: (Ala-Gly[Arg^8]) vasopressin and Val-Asp[Arg^8] vasopressin.[33] These have higher natriuretic/pressor ratios than AVP; the biological findings were also confirmed in a synthetic product.[34]

Possible additional biological roles for vasopressins were suggested by a report that the hormone inhibits binding of epidermal growth factor.[35] Following the growing evidence of a role of vasopressin in memory function,[36] a double blind study of normal and cognitively impaired volunteers revealed that DDAVP can enhance learning and memory.[37]

Among many potent antagonists synthesized to date,[38] one of the best is [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-(O-methyltyrosine]arginine vasopressin with an antivasopressor pA_2 of 8.62, but this and related compounds are not antidiuretic antagonists.[39]

Hypothalamic Release and Release-Inhibiting Hormones

Among known or predicted hypothalamic releasing and release-inhibiting factors are luteinizing hormone-releasing hormone (LH-RH), somatotropin release-inhibiting hormone (SRIF), thyrotropin releasing hormone (TRH), and the well-established but not yet synthesized factors corticotropin releasing factor (CRF), growth hormone releasing factor (GH-RF), prolactin inhibiting and releasing factors (PIF and PRF), and melanotropin related factors (MRF), all of which have been recently reviewed.[40] Another selective FSH-RH apparently unrelated to LH-RH has also been described.[41]

Luteinizing Hormone-Releasing Hormone (LH-RH, GnRH) - Of thousands of LH-RH (3) analogs synthesized since 1971, the most potent agonists (super agonists) have had D-amino acid substituents at the 6-position (D-Trp^6, D-Phe^6, or D-Ser(OBu^t)^6),[42] which when combined with the Fujino modification at the C-terminus[43] (ProNHC$H_2CH_3^9$), have yielded analogs with

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
1 2 3 4 5 6 7 8 9 10

3

potencies nearly two orders of magnitude greater than LH-RH.[44] A recently prepared [D-His(Bzl)^6,$ProNHEt^9$] LH-RH agonist is even more (210x) potent.[45]

While early antagonists, such as des-His^2 analogs, inhibited LH release at ratios of 3000:1 (antagonist/LH-RH), recent progress has been much more dramatic, with D-Trp^6 substituents and substantial N-terminal perturbations, antagonists at least 10^4 more potent have been developed. Key breakthroughs were the initial 3x enhancement of an N-terminal D-$pGlu^1$ instead of L-pGlu,[46] the further evolution of extended N-terminal[47] and Ac-X^1 replacements,[48] the use of D-Trp^3 substituents,[49] and the finding that aromatic halogenation can also yield significant improvements.[50] Thus, an [Ac-D-Phe^1,D-pClPhe^2,D-$Trp^{3,6}$]LH-RH was fully active in the rat antiovulatory assay at 62 μg/rat[50] and the substitution of Ac-dehydroproline at the one position decreased the dose required for full activity to 10 μg/rat.[51] While D-Phe^1 and D-Glu^1 replacements have proven more active than their L-counterparts, in the case of Ac-Ala^1 [52] and Ac-Pro^1,[53] the L-derivatives proved far more active. Interestingly, the [Ac-Gly^1,D-pClPhe^2,D-$Trp^{3,6}$]LH-RH derivative, with neither a D nor an L 1-position residue, was highly active at 10 μg/rat in the antiovulatory assay,[54] while an [Ac-Gly^1]LH-RH analog retains only about 1% of its

parent's agonist potency.[55]

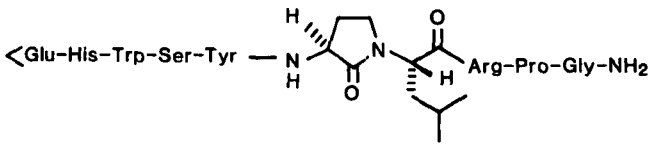
A disappointing and non-additive relationship was discerned when the Fujino C-terminal modification was introduced into antagonists. Although active in vitro at a 1:5 ratio (antagonist/LH-RH), an early [D-Phe^2,D-Ala^6, ProNHCH$_2$CH$_3$^{9}]LH-RH analog was inactive in vivo, even at 750 μg.[56] Other modifications showing poor in vitro/in vivo correlations include a 1-cyclopentylcarboxyl[57] and the 1-2 amide bond replacement, CH_2S,[58] suggesting that lipophilicity and peptide transport might be involved here. Yet the binding of Fujino-modified antagonists to pituitary homogenates is less than their non-modified GlyNH$_2$^{10} counterparts,[59] while the superagonists do show the increased binding suggestive of the observed potency improvement.

In general, binding of analogs of LH-RH toward both pituitary and gonadal receptors shows a good parallel with biological activity,[59,60] and there is convincing evidence that the anterior pituitary and ovary (of the rat) have similar LH-RH binding sites.[61] Direct gonadal effects of LH-RH and agonists continue to be supported by numerous studies in male[63] and female species,[64] and the "paradoxical" antifertility effect seen with the superagonists has been attributed to such a direct effect through inhibition of ovarian progesterone production[65] and down regulation[66] (reduction of testicular or ovarian receptor concentrations.)

Clinical trials with various superagonists have proliferated, and, through a more sophisticated, pulsatile administration of hormone analogs, usually at two-hour intervals through a mini-pump, some success has been achieved in treatment of precocious puberty.[69] Substantial progress in male endocrinological control may be anticipated in view of reports on 1) the clinical use of LH-RH analogs in human hypogonadotropic hypogonadism,[70] 2) the promising evidence of synergism observed with superagonist and testosterone coadministration,[71] and 3) the prospect that such coadministration will restore testicular weights (and prevent undesired androgen-deficiency symptoms) while maintaining the antifertility effect of disrupted tubular function.[72]

With potent antagonists, such as [Ac-dehydroPro^1,D-pClPhe^2,D-Trp^{3,6}, N$^{\alpha}$-MeLeu^7]LH-RH, male rats have shown decreased testosterone production and impaired spermatogenesis.[51] In both males and females, dosage and mode of administration will prove crucial. For example, while LH-RH antagonists have successfully decreased endogenous LH levels in chimps,[73] no accompanying antiovulatory activity was observed under the regimen used.

Among the more unusual analogs synthesized recently has been the γ-lactam-bridged, conformationally constrained analog 4.[74] This compound proved at least two-fold more active than LH-RH, and provides added support for a 6-7 bend conformation.



4

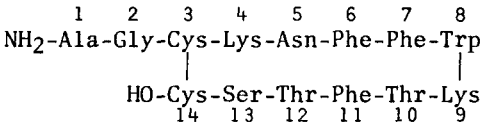
Also of interest is a dehydroalanine (Dha)-containing LH-RH agonist, [Ile^3,Dha^4,Leu^5]LH-RH, which upon cleavage, yields two new fragments, an

agonist, pGlu-His-Ile-$CONH_2$, and an antagonist, C-terminal fragment.[75] Short fragments of the superagonist [D-Ser(Bu^t)^6,Pro$NHCH_2CH_3$^{9}]LH-RH retained activity with the C-terminal tetrapeptide D-Ser(Bu^t)-Leu-Arg-Pro$NHCH_2CH_3$ showing about 1% of the parent LH-RH activity.[76]

As with other hypothalamic peptides, extrapituitary activities are the rule rather than the exception, and LH-RH-related material has been found in the rat pancreas.[77] In a study of the potential significance of this finding, LH-RH was found to inhibit glucose-induced insulin release (in vitro) at 10^{-8} and 10^{-10} M but not at 10^{-6}.[78] Thus, LH-RH joins somatostatin and TRH as potentially important regulators of pancreatic and GI function.[79]

Degradation of LH-RH by various exo- and endopeptidases has been documented to cleave the 1-2, 2-3, 6-7, and 9-10 bonds.[80] The degradation and activation of peptide hormones have recently been reviewed.[81] Reviews have also appeared summarizing developments in the use of LH-RH analogs as contraceptive agents, in both males and females[82-83] and as carcinostatic agents.[83]

Somatotropin-Release Inhibiting Hormone (Somatostatin, SRIF) - Most somatostatin analog studies have continued to focus on structures that separate the diverse biological functions, especially those specific for glucagon inhibition for potential diabetic therapy. Studies with analogs have demonstrated that selectivity for glucagon, insulin, and growth hormone receptors in the pancreas and pituitary can be achieved, and further that the nature of a given inhibitory response by an analog may be stimulus-dependent.[87] (See also Chapter 17 on Diabetes Mellitus.)

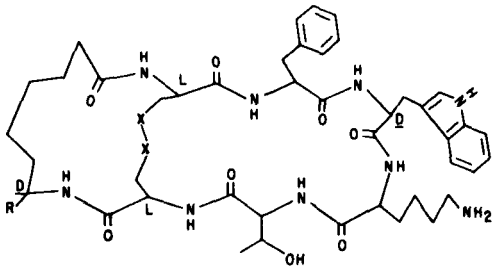


5

Recent advances in SAR studies have come from the synthesis of smaller ring analogs[7e] or rigid polycyclics,[84] as well as the more traditional amino acid substitutions, especially with novel electronically perturbed replacements. Following the report of a [Phe^4]SRIF, which is a potent and selective inhibitor of growth hormone release,[85] a later investigation involving ring-substituted Phe^4 substituents yielded a structure, [pNH_2-Phe^4,D-Trp^8]SRIF, that is 15x more active (in the pituitary) than somatostatin.[86] Potential clinical interest in compounds with selective glucagon inhibition should be enhanced by the finding that [Ala^2,D-Trp^8,D-Cys^{14}]SRIF suppressed arginine-induced glucagon release in rats while actually increasing insulin release.[87] It is significant that this compound, unlike somatostatin and several other analogs, enhanced glucose-induced insulin release. Several glycosylated somatostatin analogs were synthesized and were found to be 100-fold less active in vitro, but approximately as active as SRIF in vivo, presumably through enzymatic liberation of the free peptides.[88]

Further refinements of the polycyclic analogs of the Merck group,[84] involving replacement of a disulfide bridge by a carba link, failed to improve potencies but did yield structures with enhanced resistance to biodegradation.[89] The bicyclic disulfide bridge analog, 6a, demonstrated inhibition of release of glucagon, insulin and growth hormone with potencies of 2.7, 3.5, and 1.2, respectively (somatostatin = 1), and

showed increased resistance to trypsin degradation and longer duration of action relative to somatostatin, but the carba-bridged structures were less active in all three assays. Nevertheless, the high activities of compounds 6a and 6b, which contain only four of the original SRIF residues, provide additional support for a 7-8-9-10 turn in SRIF.



6 a) R=H, X=S b) R=H, X=CH_2

Somatostatin analogues with numerous deletions and substitutions have led to reasonably potent and selective analogs. A highly modified dodecapeptide, [des-Ala^1,des-Gly^2,His^{4,5},D-Trp^8] somatostatin (7) was found to selectively inhibit growth hormone and glucagon.[90] This structure and other smaller ring somatostatin analogs begin to resemble cyclic LH-RH derivatives, one of which (8) had earlier been shown to possess more than 1% LH-RH activity.[91] In fact it was initially believed that 7 possessed antifertility properties characteristic of LH-RH superagonists. However later study showed the LH-RH activity to be due to minor contamination by an extremely potent LH-RH superagonist.[90b]

Cys-His-His-Phe-Phe-D-Trp
| |
Cys-Ser-Thr-Phe-Thr-Lys

7

β-Ala-His-Trp-Ser-Tyr
| |
Gly-Pro-Arg-Leu-D-Ala

8

Another major development was the isolation and sequence determination of a putative somatostatin prohormone 1-28 (9).[92] This was confirmed

NH_2-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH
15 16 17 18 19 20 21 22 23 24 25 26 27 28

9

by the synthesis of the octacosapeptide by at least two groups.[93] Both ovine and porcine hypothalami yield identical structures which are 5x more effective in insulin inhibition (weight basis - or about 10x more active on a molar basis) and are 3x longer-lived than somatostatin with respect to in vivo release of growth hormone. Progress toward isolation of still larger somatostatin precursors was reported with one rat hypothalamic fragment of approximately 12,000 daltons possessing 35% of the potency of somatostatin.[94] A somatostatin analog isolated from catfish (pancreas) had the 22 amino acid sequence NH_2-Asp-Asn-Thr-Val-Arg-Ser-Lys-Pro-Leu-Asn-Cys-Met-Asn-Tyr-Phe-Trp-Lys-Ser-Ser-Thr-Ala-Cys-OH and full GH inhibition in rat pituitary cells.[95]

Somatostatin may play a role in several unrelated disease states. Altered somatostatin metabolism was suggested by variations in half-lives in hepatic failure ($T_{\frac{1}{2}}$ of 3.5 min) and acromegaly ($T_{\frac{1}{2}}$ of 1.7 min) as compared to normal degradation of 2.1 ± 0.2 min.[96] Autopsies of victims

of Alzheimer's disease showed reduced somatostatin-like immunoreactivity.[97] Intriguingly, an RIA study of toad urinary bladder layer responsive to antidiuretic hormone revealed the presence of somatostatin.[98]

Thyrotropin Releasing Hormone (TRH; TRF; thyroliberin) - TRH or pGlu-His-Pro-NH_2 has been less successfully modified than other hypothalamic peptides, although an N^{τ}-methylated analog [His(N^{τ}-Me)^2]-TRH had enhanced potency.[99] A particulary potent TRH analog, pGlu-His-3,3-dimethylprolineamide, was a 10-200x better antidepressent than TRH in several assays and was said to be more metabolically stable to *in vivo* inactivation.[100] A similar analog, pGlu-His-*trans*-3-methylprolineamide, also had improved CNS but not endocrinological activities relative to TRH.[101]

Structural Studies on Hypothalamic and Other Peptides

The recent proliferation of amide bond replacements has emerged as one of the more important new approaches to analog design and synthesis. Early work on dimethylene and ester linkages has been largely superceded by more isosteric and/or enzyme resistant links; these are intended to mimic the bioactivity profiles of hormones or inhibitors while increasing their notoriously short biological half-lives.[102] Several such replacements that have been incorporated in hypothalamic hormones and other small biologically active peptides are considered below.

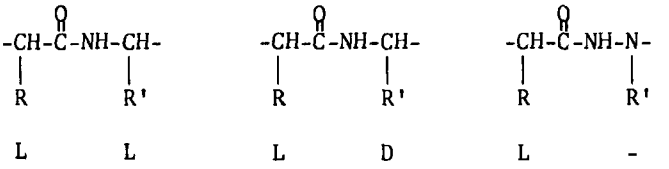
Most amide bond "surrogates" involve the deletion of the CONH function and replacement by such moieties as "retro" (NHCO),[103] alkylidene (CH=CH),[104] or ketomethylene ($COCH_2$) groups.[105] The designation ψ[] has been suggested[106] to simplify nomenclature in this area whereby ψ represents *any* amide replacement and the replacing group (e.g., $\psi[CH_2S]$ or ψ[*cis*-CH=CH] is enclosed parenthetically. The amide replacement, $\psi[CH_2S]$, has been incorporated into numerous LH-RH agonists and antagonists[58] at a variety of sites. An LH-RH agonist with a Gly$\psi[CH_2S]$Leu$^{6-7}$ substitution was virtually inactive, while a Tyr$\psi[CH_2S]$Gly$^{5-6}$ agonist retained 2-5% of the parent's activity. An LH-RH antagonist [Ac-D-Phe$\psi[CH_2S]$D-Phe$^{1-2}$,D-Trp$^{3-6}$]LH-RH was virtually as active as its corresponding amide-linked congener *in vitro* although not *in vivo*.[63]

Other replacements have likewise proven very site-dependent, with a Gly$\psi[CH_2NH]$Gly$^{2-3}$ leucine enkephalin being far less active than a Tyrψ $[CH_2NH]$Gly$^{1-2}$ derivative;[107] this was interpreted as circumstantial evidence in favor of a functional role for the 2-3 amide bond, but a newly prepared active Gly$\psi[CH_2S]$Gly$^{2-3}$ derivative makes this less likely.[106] Enkephalins have been favored hosts for amide replacements due to their synthetic accessabilities, and recent communications from two groups report syntheses of ψ[CH=CH] analogs.[104] A Tyrψ[*trans*-CH=CH]Gly$^{1-2}$ Leu-enkephalin analog retained ~50% of the parent (Leu-enkephalin) activity, while a Glyψ[*trans*-CH=CH]Gly$^{2-3}$ replacement in [ProNH_2^5] enkephalin had 0.1% activity in the guinea pig ileum assay compared to its amide counterpart.[108]

A backbone thioamide replacement, ψ[CSNH], was incorporated within the oxytocin "tail" as a C-terminal thiocarboxamide with drastic loss of activity.[109] However, the introduction of this modification to probe such features as intramolecular hydrogen bonding can now be more readily accomplished by the use of convenient new thionation reagents.[110] As to potential amide nitrogen replacements, few $\psi[COCH_2]$ replacements have yet been reported, other than the peptide ACE inhibitor discussed below.

N-methylated amino acids represent an additional class of amide bond modifications that have both conformational and stabilizational utility. Applications leading to more potent analogs of LH-RH, both agonists and antagonists, have been reported with [N-MeLeu^7] substitutions.[111] However, a series of N-methylated bradykinin analogs, patterned after potent and enzymatically resistant angiotensin and enkephalin N-methyl derivatives failed to give any analogs with increased activity.[112]

Three other approaches representing peptide backbone modifications that do not involve the amide bond, but nevertheless do affect (reduce) enzymatic degradation are 1) aza modifications, 2) α,β-dehydroamino acids, and 3) D-amino acids. The latter, much more synthetically accessible, have been widely used to increase potency and stability, as numerous hypothalamic and other peptide applications demonstrate. A particularly stable and highly potent α-MSH derivative, [Nle^4,D-Phe^7]-α-MSH, was prepared partly as a result of early reports that heat-alkali treated α-MSH preparations yielded partially racemized derivatives with increased activity.[113]

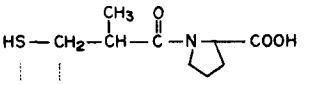


Aza analogs (10c) do not permit a chiral residue and can thus be considered as intermediate in conformational orientation between D and L amino acids.[114] Thus, their utility might be best served as replacements for a glycine residue or in positions where either D or L amino acids can be accommodated. Aza replacements have been incorporated within enkephalins, LH-RH and, more recently, somatostatin, with widely varying results. At least one LH-RH antagonist, [D-Trp^6,AzGly^{10}]LH-RH, was found to be more potent than the corresponding amide structure. A somatostatin analog with the 6-position phenylalanyl residue replaced was virtually inactive, suggesting the importance of this aromatic ring orientation.[115]

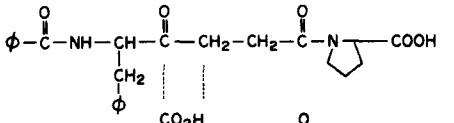
Another potentially important class of backbone modifications is represented by α,β-unsaturated amino acids, which may confer enzyme resistance to peptides thus substituted.[116,117] Several analogs of peptides, such as methionine enkephalin and an angiotensin converting enzyme inhibitor, containing a trans-dehydrophenylalanine residue have been prepared and shown to possess high biological activities.[117]

Various other amide bond modifications have been introduced into oligopeptide enzyme inhibitors. Thus, the potent angiotensin converting enzyme inhibitor captopril (11a) and second generation candidates may be viewed as amide bond replacements with ψ[HSCH$_2$],

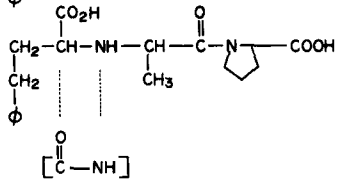
11a[118]



11b[105]



11c[119]



$\psi[CH(CO_2H)NH]$ and $\psi[COCH_2]$ surrogates. Compared to an I_{50} of 0.30 μm for captopril,[118] structures 11b and 11c have values of 0.07 μm[105] and 0.01 μm,[119] respectively. The proliferation of backbone modifications (including α-methyl amino acids,[120] etc.) and amide bond replacements will no doubt continue as the need for orally active peptide derivatives increases.

Conformational Studies - Biologically relevant solution conformations of peptide hormones continue to be probed by a great variety of methods. For example, an x-ray structural analysis of TRH was performed on its tartrate; an apparently weak hydrogen bond was discerned involving the His π-NH and the C-terminal carbonyl group.[74] Several cyclic hypothalamic peptides provide useful candidates for magnetic resonance studies due to more rigid, and definable, preferred conformations. NMR studies of oxytocin agonists and antagonists, undoubtedly the most extensively studied structures, probed differences that might rationalize factors leading to inhibitors; such antagonists as [penicillamine^1]-oxytocin and [Pen^1,Phe^2,Thr^4]oxytocin proved consistently more conformationally restricted, as assessed by relaxation studies[121] and rotamer population analysis.[122] As a result, the tyrosine-2 residue, of critical importance in the cooperative model, if unreplaced, is unable to adopt required conformations leading to favorable receptor interactions in these oxytocin antagonists.[27]

Complete proton nmr assignments for somatostatin were reported, and, in contrast to evidence from SAR studies, coupling constants incompatible with either a 7-8-9-10 or a 8-9-10-11-β-bend were found[123] (however, it cannot be excluded that a 7-10-β-bend conformation is a minor but essential one for the binding and/or transduction event).

Carbon-13 nmr studies of somatostatin and oxytocin analogs at natural abundance were reported.[124,125] Carbon-13 enrichment was used to probe the hindered rotation of oxytocin's tyrosine-2 residue while complexed to neurophysin I, and a second weaker binding mode (with rapid ring rotation) was detected.[126] The conformation of free oxytocin was also the subject of a total semi-empirical analysis which was largely compatible with previous nmr studies and with previously proposed H-bonded structures (but with a relatively free "tail").[127] Raman and CD studies[128] and a C-13 nmr study[125] with many neurohypophyseal hormones have been carried out and support the generalization that there are far more conformational similarities than differences among the various oxytocin-related agonists, even those with widely varying potencies. In contrast, antagonists appear to be consistently less flexible structures.

A Raman study of somatostatin correlated with a β-pleated sheet for the ring, and a less ordered structure for analogs such as [D-Trp^8] and [D-Cys^{14}]SRIF.[129] An nmr investigation of somatostatin was also consistent with rapidly interconverting conformers in aqueous solution, as was a study of SRIF in an oriented polyoxyethylene film,[130] which suggested a non-ordered, non-hydrogen bonded model. In view of the apparent conflict between stable, β-turn structures proposed for many hypothalamic peptides, and the consistent lack of support found for such structures as assessed by various physical studies, it seems clear that the predominant solution conformers, especially for linear peptides, are not necessarily the biologically relevant ones. Further support for this view comes from empirical energy calculations on small ring analogs of SRIF; two conformations were proposed, one with a type II' β-turn, and a second, possibly representing a new conformer, which forms upon binding to receptor.[131]

The importance of β and γ-turns in peptides and proteins is also indicated by several timely reviews;[132,133] more specific reviews on hypothalamic peptide conformational studies have also appeared.[134]

Peptides as Neurotransmitters

The role of peptides as neurotransmitters, the subject of numerous reviews[135,136] and conjecture, is supported by evidence that such peptides as somatostatin and TRF are synthesized in many areas of the brain besides the hypothalamus.[137] Nearly all of the evidence indicates that peptides of the gut are found in the brain and vice versa.[138] The startling discovery that gastrin or pentagastrin, responsible for gastric acid secretion in the stomach, is not only found in the brain but can induce stomach gastric acid secretion following intracranial (hypothalamic) injection of as little as 100 picomoles[139] adds to the intrigue.

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Chapter 20. Arachidonate Lipoxygenase

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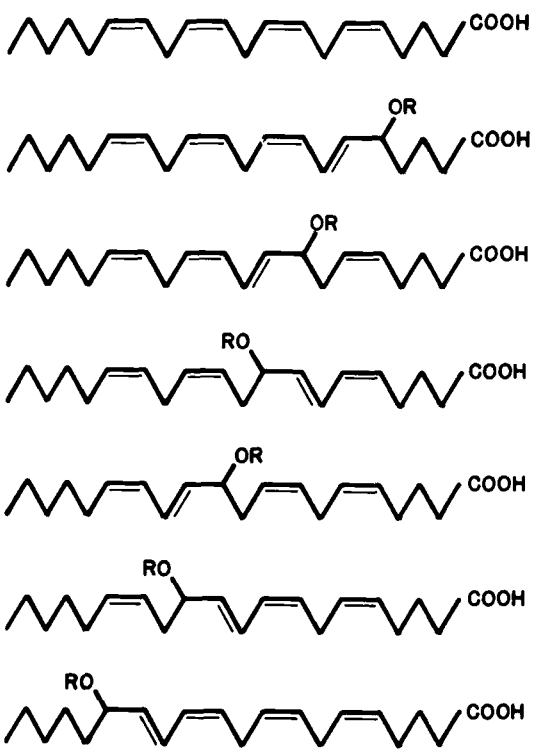
In the few years since the identification of mammalian lipoxygenases, the significance of these enzymes has been dramatically documented. This review will highlight recent developments in the understanding of these systems in light of an historical perspective.

For consistency, the nomenclature of Samuelsson[1,2] will be used throughout the review.

INTRODUCTION

Lipoxygenases are enzymes that catalyze the hydroperoxidation of polyunsaturated fatty acids, esters, alcohols, etc., containing a cis-cis-1,4-pentadiene system; the products contain 1-hydroperoxy-2,4-trans, cis-pentadiene fragments.[3] The enzymes belong to the class known as dioxygenases that characteristically incorporate two atoms of molecular oxygen into a substrate, e.g., $R+O_2 \rightarrow R-O_2$.[4] Arachidonic acid (AA) 1, contains three overlapping cis, cis-1,4-pentadiene moieties and therefore can give rise to six primary lipoxygenase products (hydroperoxyeicosatetraenoic acids, HPETEs) 2-7. Prior to 1974, only plant lipoxygenase was known, a crystalline enzyme having been obtained (but incorrectly characterized) from soybeans in 1947.[5] Plant lipoxygenases have been reviewed recently[6] and the proceedings of a symposium have been published.[7]



	R=OH	R=H
1		
2		2a
3		3a
4		4a
5		5a
6		6a
7		7a

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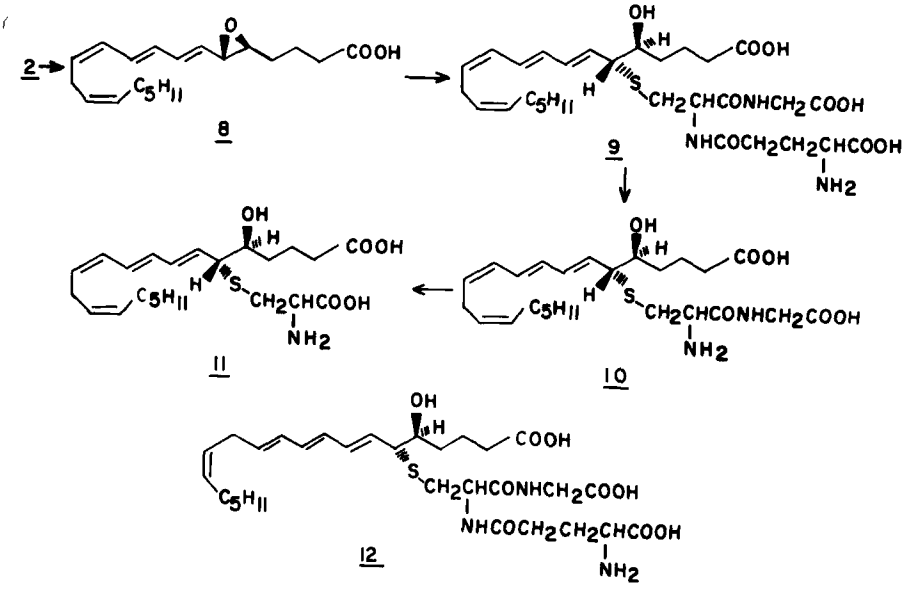
I. The Arachidonate Lipoxygenase Cascade

In 1974, Hamberg and Samuelsson isolated L-**6**, after incubation of labeled AA with homogenized human platelets and attributed its formation to a novel lipoxygenase; the reduced hydroxyeicosatetraenoic acid (HETE), L-**6a** (L-12-HETE), was also isolated.[8] From guinea pig lung homogenate incubated with AA was isolated **5a** (11-HETE), **6a** (12-HETE) and **7a** (15-HETE), and a reductive step after lipoxygenase was postulated.[9,10] Guinea pig lung and spleen homogenates produced at least ten times as much 12-HETE when incubated with AA as did homogenates of kidney, stomach, liver, heart and whole blood.[11] The term arachidonate lipoxygenase was coined shortly thereafter when the enzyme isolated from platelets of various mammals displayed higher conversion rates for AA than for a variety of other fatty acids containing the cis-cis-1,4-pentadiene fragment.[12] The subject of arachidonate lipoxygenase was later reviewed.[13] A novel lipoxygenase was considered responsible for the formation of D-**2a** (D-5-HETE) from AA-treated rabbit polymorphonuclear leucocytes (PMNL).[14] Human neutrophil-derived lipoxygenase was used to first demonstrate the production of (among other products) 8-HETE (**3a**) and 9-HETE (**4a**) from AA.[15]



Dihydroxyeicosatetraenoic acids (di-HETEs) were isolated after incubation of suspensions of rabbit peritoneal PMNL with AA,[16,17] and the unstable **8** (leukotriene A_4) was proposed as the reactive intermediate.[18] Further oxygenated trihydroxyeicosatetraenoic acids presumably arose from **6** via an epoxy-hydroxy product in washed human platelets incubated with AA.[18,19] Platelets from selenium-deficient rats produced large amounts of trihydroxy acids presumably as a result of decreased reduction of **6** to **6a** by selenium-dependent glutathione peroxidase.[20]

The relationship between slow reacting substances (SRSs) and the arachidonate lipoxygenase pathway in rat basophilic leukemia cells (RBL-1) was proposed in 1977.[21,22] Reviews on SRSs have recently appeared.[23,24] Murine mastocytoma cell-derived SRS[25] was later shown to be leukotriene C_4 (**9**).[26,27] Later studies determined the amino acid portion of leukotriene D_4 (**10**)[28,29] and demonstrated the conversion of leukotrienes C_4 and

D_4 to leukotriene E_4 (**11**) in human plasma[30] and in RBL-1 cells.[31] Publications from several groups confirm **10** as a major component of SRS-A.[32-35] The 11-trans-leukotriene C_4 (**12**) (leukotriene "C-2") was detected as a naturally occurring SRS in murine mastoma cells.[36] Several reviews on leukotrienes are available.[37-43]

Syntheses of leukotrienes A_4 (ester),[44-47] B_4,[48,49] "C-1",[43,50-52] "C-2",[53] D_4[53] and E_4[54] have appeared.

II. Location of the Enzyme

In addition to human platelets,[8,12] guinea pig lung[9] and spleen[11] homogenates, rabbit PMNL[14], human neutrophil[15] and rat RBL-1 cells[21] already mentioned, other tissues have been reported to display lipoxygenase activity against a variety of substrates. In an early study, activity attributed to "lipoxygenase" in rabbit reticulocytes paralleled the release of malonyl dialdehyde.[55] The latter fragment is associated with cyclooxygenase pathways.[56] More recently, a non-globin "lipoxygenase" has been isolated from rabbit reticulocytes and identified with the respiratory inhibitors of the reticulocyte.[57,58] Involved psoriatic epidermal cells showed higher levels of lipoxygenase activity than did uninvolved cells.[59] The high levels of AA in involved tissue were unexpected and paralled the concentrations of HETE.[60] A reduction in the cAMP/cGMP ratio in involved epidermis was noted,[59,60] and guanylate cyclase activity was observed to be stimulated by AA and 12-HETE in both involved and uninvolved tissue.[61] Rabbit alveolar macrophages were reported to produce hydroxy fatty acids from labeled AA, but the products were not identified. The production of the hydroxy acids could not be prevented by indomethacin (see Section VI B).[62] In a preliminary report, 11-HETE (**5a**) was tentatively identified as a lipoxygenase product from rat mast cells.[63] Products obtained from rat testes microsomes and linoleic acid were attributed to "lipoxygenase-like" and hydroperoxide isomerase enzymes.[64,65] Similarly, ram seminal vesicle microsomes, especially under the influence of added ascorbic acid, produced 11-HETE (**5a**) and 15-HETE (**7a**), as well as products of the cyclooxygenase pathway.[66] Lipoxygenase pathways were demonstrated in mouse[67,68] and rat[69] peritoneal macrophages, with leukotriene B_4 accounting for 80% of the activity generated in the latter preparation. In another study with mouse and guinea pig peritoneal monocytes, 8-,9-,11-,12- and 15-HETE were detected in varying amounts by coupled gas chromatography-mass spectrometry (GC-MS); few prostaglandin (PG) products were formed.[70] A similar analytical technique was used to detect HETE in the cerebral cortex of rats and, thus, to implicate a centrally located enzyme in that species.[71] Mesothelium-rich tissues from rabbit (pericardium, pleura and peritoneum) generate 12-HETE along with prostacyclin, and an organ-environmental control function was postulated for these metabolites.[72] Neoplastic tissues continue to be of interest for investigations of arachidonate metabolism, and the lipoxygenase activity of VX_2 carcinoma tissue appears to be localized in the microsomal fraction.[73] The SRSs released on perfusion of cat paws with compound 48/80 have been identified as leukotrienes D_4 and E_4.[32] In studies with human tissues, peripheral blood PMNL treated with labeled AA and ionophore A23187 (see Section VI A) produced 5-HETE, 15-HETE, 5,12-di HETE and leukotriene A_4.[74,75] Leukotriene C_4 and 11-trans-leukotriene C_4 (leukotriene "C-1" and "C-2") were similarly isolated.[76] Lipoxygenase in human lung has been studied as a function of SRS release,[77] and 15-HETE,[78] as well as leukotriene "C-1" and D_4,[79] have been identified. Knee synovial fluid from patients with traumatic and degenerative arthropathies or various arthritides have yielded 5-HETE and 5,12-di HETE.[80]

III. Characterization of the Enzymes

Plant lipoxygenase has been known and characterized for some time and recent reviews have already been noted.[6,7] The general topic of oxygenases in fatty acid metabolism was covered earlier.[81] A soluble lipoxygenase with a pH optimum between 8 and 9 was first isolated from the 100,000 g supernatent of fractured bovine blood platelets;[12] gel chromatography gave a major (MW>300,000) and a minor (MW 100,000) fraction with lipoxygenase activity.[13] A heat-labile enzyme was obtained from the microsomal fraction of human platelets.[82] Enzyme from human platelet cytosol was fractionated into two species one of which also contained peroxidase activity[83] capable of converting HPETE to HETE; an essential sulfhydryl group and an essential metal ion were suggested.[84] As is the case with plant lipoxygenase,[85] evidence points to a requirement of ferric iron for platelet lipoxygenase.[86-89] By contrast, platelet cyclooxygenase is dependent on ferrous iron and a model for the AA-heme interaction has been proposed.[90] A lipoxygenase of MW of ca. 13,000 from Fusarium oxysporum contained protoheme IX and required Co^{+2} for stability; the enzyme was extensively characterized.[91-94] The "lipoxygenase-like" enzyme from rat testes was purified to near homogeneity by affinity chromatography.[64,65] The lipoxygenase substrate structural requirements have been recently reviewed.[95]

IV. Assays

Numerous techniques for the determination of levels of plant lipoxygenase activity have been developed and many are included in a recent review.[96] A simple test paper detection system has been described.[97] Modifications of the assays for plant enzyme have been used with fungal and mammalian lipoxygenase,[67,91] and an AA-induced aggregation of PMNLs has been proposed as a simple bioassay.[98] The thiobarbituric acid reaction has been adapted to measure lipoxygenase activity,[99] but the response in this assay may in part be due to PG endoperoxides.[100] A variety of radiochemical and chromatographic procedures have been developed for the separation and quantification of AA lipoxygenase products, and the reader is referred to the references of Section I for many examples; some special techniques have been noted.[101-103] High pressure liquid chromatography (HPLC) on a cation-exchange resin was used to separate soybean lipoxygenase-1 into five subfractions,[104] and reverse phase techniques have been used extensively for purification of mammalian SRS (cf. ref. 23 and 151, and references cited therein). Coupled GC-MS has been extensively used for identification and assay of lipoxygenase products and lead references are cited.[70,105,106] A NMR shift procedure for determining the enantiomeric composition of hydroperoxides formed by plant lipoxygenase has been published.[107] A radioimmunoassay has been developed for 12-HETE.[108] Highly specific antibodies to a conjugate of platelet-derived 12-HETE and human serum albumin were raised in rabbits, and assays using the technique were in agreement with radiochemical quantification.[109]

V. Biologic Consequences of the Activity of the Lipoxygenases

When the prostaglandins and other metabolites of arachidonic acid were implicated in inflammatory processes, some interest focused on polymorphonuclear leukocyte fatty acid metabolism, as these cells are involved in the pathophysiology of inflammation.[43] Since the early discoveries of Borgeat, Hamberg and Samuelsson (see above), much work has been directed toward the products of the arachidonate lipoxygenase cascade. Although the consequences of the synthesis and release of the products of this

cascade have yet to be fully appreciated in terms of broad biologic implications, the chemical identification of the SRSs presumably has solved a significant forty year old mystery. The biology of these materials promises to be at least as significant as that of the products of the cyclooxygenase pathway of AA metabolism, which forms the endoperoxides, prostaglandins, thromboxanes and prostacyclin. Thus, the lipoxygenation of AA by a variety of cell types can produce substantial consequences. These may be broadly classified as spasmogenic in the case of 5-HPETE and the leukotrienes, and chemotactic in the case of the HETEs and di-HETEs which enhance PMNL migration.

Determining the spasmogenic effect of the lipooxygenase products of AA metabolism on tracheobronchial smooth muscle has been one of the more important steps in the research on SRSs.[39,110-112] The release of the anaphylactic mediator, SRS-A, from guinea pig lung is potentiated by unsaturated fatty acids. However, while exogenous AA is incorporated into pulmonary phospholipids, apparently it is not incorporated directly into SRS-A.[113] The enhanced release of the mediator of anaphylaxis may be due to a sequential displacement of AA which is then metabolized by the lipoxygenase.

The biological profiles of leukotriene C_4, D_4 were partially characterized.[114] In rodents, the lipoxygenase pathway was convincingly implicated in SRS biosynthesis.[21,27] Apart from spasmogenic effects, SRS-A liberated during airway anaphylaxis impairs mucus transport.[115]

Lipid molecules have been implicated as chemotatic factors for a number of years. Some PGs have been associated with leukocyte chemotaxis, as have the blood platelet lipoxygenase produced HETEs.[116] These monohydroxy eicosatetraenoic acids have been implicated in human neutrophil[117,118] and eosinophil migration,[119-122] and neutrophil degranulation.[123] Thus, Goetzl and others have concluded that leukocyte lipoxygenase products of AA have the capacity to amplify the cellular component of inflammatory reactions.[124] HPETE, the labile intermediate, and HETE have additionally been implicated as possible endogenous regulators of prostacyclin (PGI_2) biosynthesis, thus perhaps serving a physiological role in the regulation of vascular tone.[125] As to whether vascular PGI_2 synthesis is likely to be specifically influenced by the platelet derived lipoxygenase product HPETE is not established.[126] Further, SRS-A has been reported to sensitize the heart to the chronotropic and arrhythmogenic effects of histamine released as a result of cardiac analphylaxis.[127]

The 5,12-di-HETE has been suggested as an important mediator in the accumlation of leukocytes in the complex inflammatory process.[80,129] Additionally, lipoxygenase products of AA metabolism are present in the conjunctiva, presumably accounting for leukocytes in conjunctional exudates[130] and the phenomena of conjunctival inflammation. HETEs and some of the products of the cyclooxygenase system may be regulators of epidermal function as HETE levels were elevated in lesional epidermis of psoriasis.[131] Further, soluble guanylate cyclase activity in psoriatic epidermis was stimulated by AA and HETE, while the activity from control normal skin was unaffected.[61,132]

Beyond these activities, lipoxygenase activity may modulate platelet functions, and a selective lipoxygenase deficiency might lead to platelet hyperfunction and myeloproliferative disorders.[133] A similar imbalance of cycloxygenase and lipoxygenase pathways has been suggested in aspirin-induced asthma.[134]

Lipoxygenase products have been implicated as regulators of neuronal excitability in the brain,[135] and a high level of HETEs in human, post-cesarean amnion has suggested a role for lipoxygenase products in the process of labor.[136]

VI. Chemical Modulation of the Lipoxygenase System

A. Stimulation

The divalent cation ionophore A23187 has been used extensively to stimulate the formation of SRS's, and recent studies have confirmed the ability of this substance to promote SRS production from a variety of tissues.[137-140] This ionophore was also reported to stimulate the production of 5-HETE and 5, 12-di-HETE from human PMNL[74] and 12-HETE from rat mast cells.[141] Addition of Ca^{+2} (but not Mg^{+2}) to the 10,000 x g supernatant of RBL-1 cell homogenates resulted in a pronounced enhancement of the formation of 5-HETE and 5,12-di-HETE but the stimulant effect could not be demonstrated with platelet 8,000 x g supernatant.[142] It had earlier been reported that Ca^{+2} enhanced and Mg^{+2} inhibited the A23187-stimulated formation of HETE in human platelets.[143] A report on the involvement of divalent cations and AA in neutrophil aggregation has appeared.[144] Sodium fluoride has been observed to enhance the synthesis and release of SRS from antigen challenged guinea pig lung preparations without bringing about the simultaneous release of histamine.[145] A similar effect had been observed several years earlier in human lung fragments challenged in the presence of the fungal metabolites cytochalasin A and B.[146]

The observation that SRS production was markedly enhanced by the addition of mercaptans to the preparations[147,148] was confirmed and extended with studies in rat peritoneal cells.[149] In this study, using a series of mercapto acids, maximum activity was located in compounds in which the -SH and -COOH groups were separated by 2 carbon atoms. Amino and alkyl substituants were also studied. Sok et al. demonstrated that L-cysteine increased SRS activity by preventing the destruction of SRS-A by aminopeptidases and not by enhancing its synthesis or release.[150] Other effects of altering mercaptan levels were studied by adding sulfhydryl scavengers to RBL-1 cell preparations. SRS biosynthesis was inhibited and the levels of other oxygenated AA products were also affected.[151]

The 12-HPETE has been found to stimulate its own production by increasing lipoxygenase activity. The studies, done in human platelet homogenates or in intact platelets, showed 8-, 9- and 11- HPETE to be much less effective.[152] A vasoconstrictor factor obtained from human plasma produced concentration-dependent increases in the levels of 12-HETE in platelet preparations.[153]

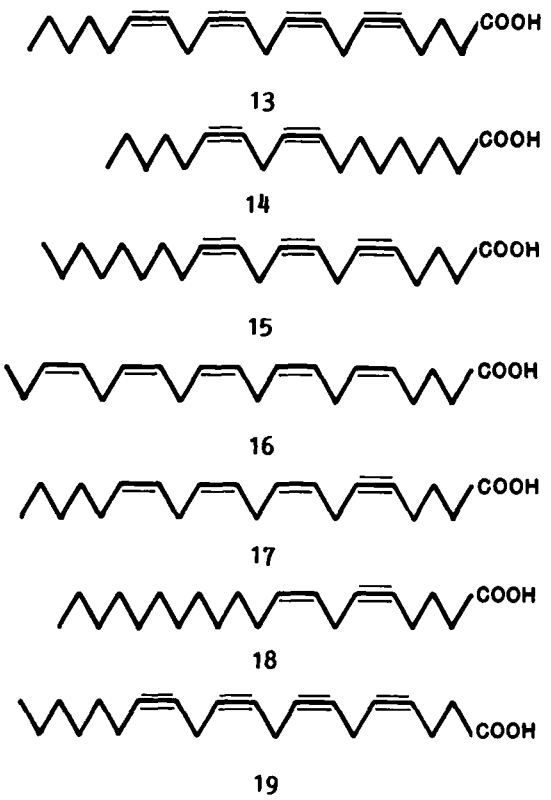
Using a rat model, Higgs et al. characterized the differential effect of the non-steroidal antiinflammatory drugs (NSAIDs) indomethacin, aspirin, sodium salicylate, phenylbutazone and flurbiprofen on leukocyte migration and cyclooxygenase activity.[154] Since phenylbutazone and sodium salicylate effected leukocyte migration without altering PG levels, the authors suggested that these drugs may potentiate lipoxygenase. NSAIDs enhance SRS release presumably as a result of diversion of AA from cyclooxygenase to lipoxygenase mediated pathways.[155,156]

Factor(s) which stimulated the formation of HETE in rat peritoneal macrophages, rabbit platelets and horse platelet homogenates were detected in the serum of adjuvant arthritic rats.[157] The material was partially

purified and had a MW 100,000.

B. Inhibition

Acetylenic and Olefinic Compounds - Blain and Shearer first studied the effects of a number of acetylenic compounds on soybean lipoxygenase.[158] Only compounds that resembled the natural substrates were effective, with 5,8,11,14-eicosatetraynoic acid (5,8,11,14-ETYA, **13**) being one of the better competitive inhibitors. The slow but irreversible inhibition of lipoxygenase by **13** was later demonstrated and an allenic intermediate was proposed.[159] Of a large number of acetylenic acids tried, only **13** and octadeca-9,12-diynoic acid (**14**) inhibited the plant enzyme.[160] Both platelet lipoxygenase and fatty acid cyclooxygenase were found to be inhibited by **13**.[8,59,161] No selectivity was shown with **13**, but 5,8,11-eicosatriynoic acid (**15**) was 10 X more potent an inhibitor of lipoxygenase than of cyclooxygenase.[162] Lectin-induced mitogenesis in human lymphocytes was inhibited by **13** and this action was attributed to its effect on lipoxygenase.[163] In horse neutrophils, **13** blocked the formation of 5-HETE from labeled AA and apparently was competitive with AA for incorporation into phosphatidic acid.[164] Histamine release from rat mast cells[165] and human basophils[166] was inhibited by **13**, and similar effects in the latter system were achieved with **15**.[167] The triyne **15** also has been reported to prevent the biosynthesis of leukotriene C_4 in mouse mastocytoma cells.[168] Compound **13** blocked the contraction of sensitized guinea pig trachea induced by antigen but not that induced by methacholine[169] and was effective in inhibiting the AA-induced contractions of lung strips from the same animal.[170] The 5,8,11,14-tetraynoic acid also was reported to block the contractions of guinea pig trachea induced by C5a, the fifth component of complement,[171] and the angiotensin- and oxytocin-induced contractions of rat uterus.[172] The effect of C5a on SRS activity in guinea pig lung had been previously noted.[173] Aerosolized **13** was shown to be effective in inhibiting IgE-mediated rhesus monkey asthma in both a standard, and a threshold antigen-challenge system.[174] This is reported to be the first agent, other than cromolyn or β-agonists, to demonstrate this activity. The animal model has many of the characteristics of human allergic asthma.[174]



13 14 15 16 17 18 19

The 5,8,11,14,17 eicosapentaenoic acid **16** appears to have a high binding affinity for platelet cyclooxygenase and lipoxygenase and inhibits

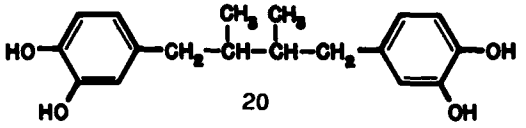
the metabolism of AA in this system.[175,176] In mouse mastocytoma cells, however, **16** is converted by lipoxygenase to leukotriene C_5.[177] Compound **16** has been suggested as a potential anti-thrombotic agent.[178,179]

The 15-HPETE (**7**) decreases lipoxygenase activity in human platelets.[180,181] At the IC_{50} concentration (for platelet lipoxygenase inhibition), 15-HETE (**7a**) had no effect on cyclooxygenase.[181]

The synthetic derivatives **17** and **18** were made as potential inhibitors of the bioconversion of AA to lipoxygenase products.[182] These materials have not been evaluated as of this writing.

From studies on a large number of acetylenic acids, 5,8,11,14-ETYA and 4,7,10,13-ETYA (**19**) emerged as the most potent inhibitors of the formation of 12-HETE in human platelets.[183,184]

Antioxidants - A large number of antioxidants, including α-naphthol, guaiacol, nordihydroguaiaretic acid (NDGA, **20**), propyl gallate, butylated hydroxytoluene (BHT), α-tocopherol (vitamin E), etc., have been examined for their effect on bovine vesicular cyclooxygenase and soybean lipoxygenase.[185] Of these, BHT and vitamin E were reported to be specific inhibitors of soybean lipoxygenase.[185] Vitamin E inhibits the conversion of AA to HETEs in human neutrophils[186] and in human and rabbit platelets;[187] the surfactant effects of the vitamin have been implicated in the mechanism.[187] Several inhibitory actions of NDGA have been attributed to its effect on lipoxygenase, e.g., on mitogenesis in human lymphocytes,[163] on anaphalactic contractile responses in guinea pig parenchymal strips[188] and on the SRS release-potentiating effect of indomethacin in human lung.[77]



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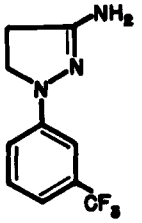
Non-Steroidal Antiinflammatory Drugs - As was noted earlier, NSAIDs tend to enhance SRS release by shunting mechanisms.[155,156] Some NSAIDs also effect AA metabolism in vitro by inhibiting the conversion of HPETE to HETE (reversibly in platelets[84,189] and irreversibly in neutrophils[190]). Reduced levels of HETE have been observed in vivo after administration of indomethacin.[191]

One of the most widely studied agents is BW-755C[192] (**21**), which was first shown to inhibit cyclooxygenase and lipoxygenase in horse platelets and inhibit leukocyte migration in a rat inflammation model.[193] A related compound (**22**) had earlier been shown to be an inhibitor of cyclooxygenase and lipoxygenase in perfused guinea pig lung.[194] BW-755C has been shown to produce a concentration-dependent inhibition of contraction of sensitized guinea pig trachea to ovalbumin[195,196] and was effective in inhibiting exudate formation and leukocyte migration in a carrageenin edema model.[197,198] Reversal of indomethacin-induced increases in leukocyte migration by



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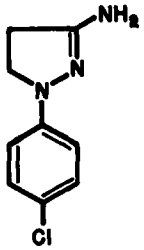

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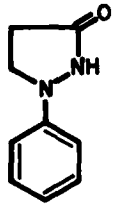

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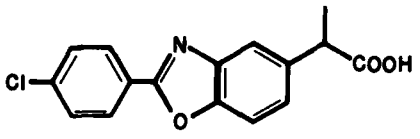
BW-755C was also demonstrated.[154,198] The formation of leukotriene C_4 and 11-trans-leukotriene C_4 in human PMNL suspensions was completely blocked by this agent,[76] and it was found to be equally inhibitory for SRS release and HETE production in rabbit PMNL.[199] Phenidone (23), a photographic reagent of structure related to BW-755C, has also been shown to be a lipoxygenase inhibitor.[77,188,200]

Benoxaprofen (24) is a weak inhibitor of cyclooxygenase but produces a dose-related decrease in lipoxygenase products from rabbit PMNL with an IC_{50} of approximately 30 μg/ml;[201] the relevance of this combined profile to the antiinflammatory activity of the drug has been reviewed.[202]



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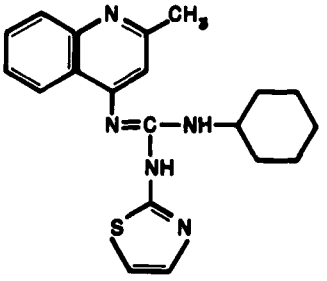

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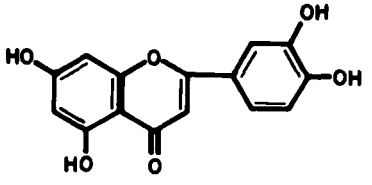
Timegadine (25), a novel, basic antiinflammatory compound is a potent, competitive inhibitor of PG synthetase and also demonstrates lipoxygenase inhibition.[203] The importance of the dual mechanisms was discussed.[204]

Miscellaneous Inhibitors - A large list of flavonoids was examined for inhibition of lipoxygenase from soybean and from rat lung and spleen and for inhibition of PG synthetase from rat renal medulla.[205] Of the several active flavonoids, **26** (IC_{50} 30 μM) and **27** (IC_{50} 33 μM) were the most potent vs lipoxygenase. Among other compounds examined for lipoxygenase inhibition were 1,5-dihydroxynaphthalene (IC_{50} 20 μM), 2,3,-dihydroxynaphthalene (IC_{50} 60 μM) and MK 447 (**28**, IC_{50} 130 μM). A comparison of **27** and 1,5-dihydroxynaphthalene using plant and rat lipoxygenases showed almost identical inhibition properties, adding evidence that most soybean lipoxygenase inhibitors inhibit animal lipoxygenase as well.[205]



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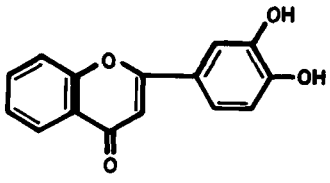

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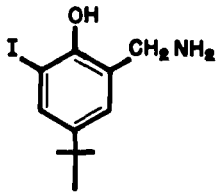

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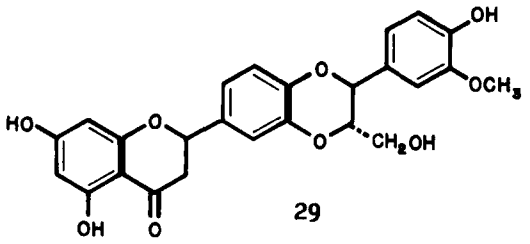
Chlorpromazine was found to be an inhibitor of soybean and horse platelet lipoxygenase.[206]

The components (**29-31**) of silymarin, the antihepatotoxic principle of the fruit of the milk thistle were shown to be non-competitive inhibitors of soybean lipoxygenase when linoleic acid was used as a sub-

strate.[207] The potencies were 30>31>29.



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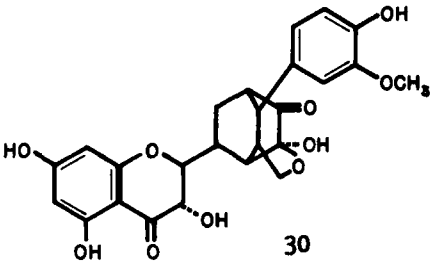

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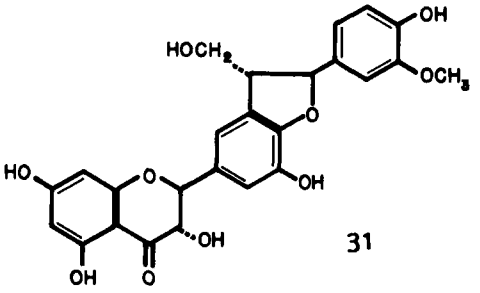

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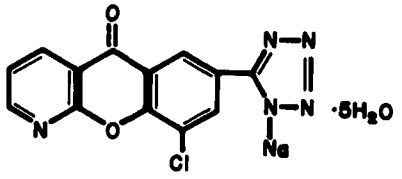
The compound Y-12,141 (32) was reported to inhibit antigen-induced release of SRS in a rat model of active bronchial anaphylaxis, but the precise step of the inhibition was not determined.[208]



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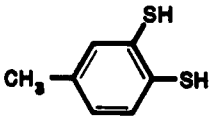

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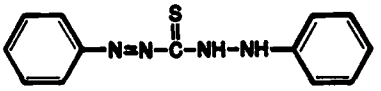

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Acetone phenylhydrazone,[209] high concentrations of glucose (but not galactose or lactate),[210] and the iron chelators 33 and 34[89] have been reported to inhibit platelet lipoxygenase. Lipoxygenase in sensitized guinea pig lung tissue is inhibited by diethylcarbamazine.[211]

An endogenous inhibitor of lipoxygenase has been postulated.[212]

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Section V - Topics in Biology

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Chapter 21. The Human Interferons

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Interferon was characterized as an antiviral protein by Isaacs et al.[1,2] in 1957. Since that time a vast literature on interferon has accumulated (see references 3-15 for reviews and general compendia). The interferons are proteins with characteristic antiviral activities and species specificities produced by various cells. Even within a species, several interferons are produced with biologically, chemically, and physically distinct antiviral activities. In general, the species specificity of an interferon provides a characteristic profile of each interferon. For example, human leukocyte interferon exhibits a high degree of antiviral activity in bovine or porcine cell cultures, whereas fibroblast interferon is hardly active and immune interferon shows no activity on these cells. Until recently, the lack of reliable definitive data on composition of the interferons was due to the failure to obtain significant amounts of any pure interferon for analysis. However, in the past few years, progress in the purification of several interferons has provided sufficient material for chemical analysis, as well as biological and physical characterization. The amino acid compositions as well as initial amino acid sequences for several human interferons have been reported. Furthermore, with the successful construction and identification of DNA recombinants containing the human interferon-coding sequences and genes, knowledge of the primary structures of several of the human interferons has emerged rapidly.

The human interferons have been classified into three distinct classes by their antigenic, biological, and chemical properties. These are human leukocyte (L), fibroblast (F), and immune (I) interferons (IF) (abbreviated IFL, IFF, and IFI, respectively). These are species that are predominantly synthesized by buffy coat leukocytes (IFL), fibroblasts (IFF), T-lymphocytes (IFI). A second human fibroblast interferon has been described.[16,17] So far, little is known about the structure of the immune interferons, but undoubtedly the structure of this entity will emerge very soon.

The interferons exhibit potent antiviral activity by which they are generally assayed. Preliminary clinical trials have indicated that human interferon is effective in a number of viral diseases.[18] Furthermore, studies have suggested that interferon may have antitumor effects on several human malignancies.[19-21] Additional clinical evaluations are in progress, but because of the difficulty of obtaining large amounts of human interferon, clinical trials have been limited. In addition, most studies with interferon have been performed with material of which less

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than 1% is human interferon.[22] However, recent achievements in purification, in determination of interferon structure and in cloning and expressing human interferon in bacteria have altered the course and rate of application of human interferon for therapeutic use. As this report is being written, initial clinical trials with purified human leukocyte interferon (recombinant species A: IFLrA) have begun as a consequence of the unique and rapid application of these new techniques in protein chemistry, as well as those in recombinant DNA technology. This report summarizes these new techniques and accomplishments.

PURIFICATION AND CHARACTERIZATION OF HUMAN LEUKOCYTE INTERFERON

Numerous attempts were made to purify interferon by conventional protein fractionation techniques[23-30] as well as by affinity chromatography with antibodies,[25-27,31,32] lectins,[33,34] polynucleotides,[27] and numerous other small ligands.[34-36] Indeed, it was reported that human fibroblast interferon[24,30] and mouse L-cell interferon[27,29] were purified to homogeneity, but no chemical composition or any characterization of these components was provided at that time. In the past few years, analysis and detection of amino acids, peptides, and proteins at the picomole level by fluorescence has been developed by Udenfriend, Stein and coworkers.[37-39] This technique was combined with high-performance liquid chromatography (HPLC) for the rapid fractionation and detection of peptides[40] up to a molecular weight of 12,000 and for the purification of human leukocyte interferon.[41,42] The methods offer a combination of high resolution and high sensitivity that is essential for the purification of proteins, such as interferon, that are present in small quantity in cell extracts or in growth media.

Purification by HPLC - Interferon was produced by incubation of human leukocytes with Newcastle disease virus.[41] The HPLC steps were reverse-phase chromatography at pH 7.5 on LiChrosorb RP-8 (octyl groups chemically bound to silica microparticles), normal partition chromatography on LiChrosorb Diol (glycerol groups on silica) and reverse-phase chromatography at pH 4.0 on LiChrosorb RP-8. Gradients of n-propanol were used for elution of interferon from these columns (Fig. 1). The overall purification was about 80,000-fold and the specific activity of pure interferon was 2-4 x 10^8 units/mg. Interferon prepared by this procedure yielded a single band of molecular weight 17,500 on polyacrylamide gel electrophoresis.

Several reports had previously described high-performance liquid chromatography of proteins, mainly on ion exchange and size exclusion columns.[43,44] However, those systems were either not commercially available or had a low capacity. With proper choice of eluent and pore size, octyl and octadecyl silica could be used for high resolution reverse-phase HPLC of both peptides and proteins. Accordingly, with n-propanol as eluent the use of Lichrosorb RP-8 (octyl silica) columns for protein fractionation was a major factor in the success of the purification (Fig. 1, Panels A, C, and D). In addition, LiChrosorb Diol, which is chemically similar to glycophase resins that have been used for exclusion chromatography of proteins, was introduced as a support for normal partition chromatography of proteins (Fig. 1B). High recoveries of interferon activity were obtained in each chromatographic step, a requirement when small amounts of initial starting material are present. Although the initial experiments were performed with leukocytes from normal donors[41,42] it was found that leukocytes from patients with chronic myelogenous leukemia (CML), who were undergoing leukapheresis to lower their peripheral white blood cell counts, were a rich source of interferon that

appeared to be essentially identical to the human leukocyte interferon purified from leukocytes from normal donors.[45] As with HPLC on the Diol

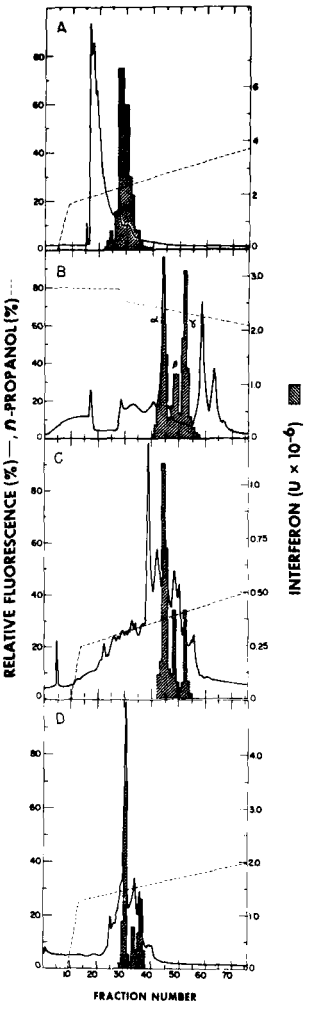

FIGURE 1. High-performance liquid chromatography of interferon. (A) Chromatography on LiChrosorb RP-8 at pH 7.5. The fluorometer scale was set to 100 and 2% of the column effluent was directed to the fluorescamine monitoring system. (B) Chromatography on LiChrosorb Diol at pH 7.5. The fluorometer scale was 30 and 2% of the column effluent was directed to the fluorescamine monitoring system. (C) Chromatography on LiChrosorb RP-8 at pH 4.0. The fluorometer scale was 1 and 5% of the column effluent was directed to the fluorescamine monitoring system. (D) Rechromatography on LiChrosorb RP-8. The conditions were similar to those of Step C. Several preparations carried through Step C were pooled (13 x 10^6 units) and applied to the last column. The gradations on the abscissa correspond to the end of the fractions. (Taken from reference 41.)

column (Fig. 1, Panel B), three major peaks of activity, labeled α, β, and γ, were observed. Although the protein profiles were almost identical, the activity profiles showed that the amount of activity under peak γ was lower in preparations from leukemic cells compared to normal

leukocytes.[45] However, even from normal leukocytes the ratio of peaks α, β, and γ varied from one preparation to another. Human lymphoblastoid interferon produced by suspension cultures of Namalva cells was purified by a combination of immunoaffinity chromatography and other methods.[46] Amino acid analysis of human leukocyte interferon purified by HPLC as described above[41] shows similarity with human lymphoblastoid interferon[45] and one of the types of mouse L-cell interferon.[47]

Human leukocyte interferon is heterogeneous and several bands containing antiviral activity ranging in molecular weight from 15,000 to 21,000 are observed on SDS-polyacrylamide gel electrophoresis.[48] Heterogeneity of human leukocyte interferon has also been observed by isoelectric focusing[49] and several types of chromatographic procedures.[25,50-52] As noted above, our initial work with high performance liquid chromatography revealed three major groups of interferon species which were labeled α, β, and γ according to their order of elution from a LiChrosorb Diol (polar-bonded phase) column.[41,45] Group γ was further resolved into several components, of which γ_2 was homogeneous and was used for the determination of molecular weight, amino acid composition, and specific activity.

Since leukocytes obtained from patients with chronic myelogenous leukemia produced high levels of interferon, these were used for preparation of interferon for resolution of the various components. The fractionation of interferon from CML cells was performed by a procedure similar to the one used for interferon from normal buffy coats. However, the amounts of the individual species of interferon varied from one preparation to another. A summary of the molecular weights of various species that were purified is given in Table I.[53] Based on the elution position, the molecular weight, and the biological properties, γ_2 was found to be identical in amino acid composition with the γ species of interferon first isolated from normal leukocytes.[41]

TABLE I. Molecular Weights of Human Leukocyte Interferons[a]

Species	*M.W.*
α_1	*16,500*
α_2	*16,200*
β_1	*16,200*
β_2	*16,500*
β_3	*21,000*
γ_1	*17,700*
γ_2	*17,700*
γ_3	*17,200*
γ_4	*21,000*
γ_5	*16,500*

[a]*The molecular weights were estimated by NaDodSO$_4$-polyacrylamide gel electrophoresis with cytochrome C and chymotrypsinogen A as molecular weight markers.*

TABLE II. Specific Activities of Human Leukocyte Interferons

Species	*Specific Activity on Bovine MDBK Cells (units/mg)*	*Specific Activity on Human AG-1732 Cells (units/mg)*
α_1	2.6×10^8	2.6×10^8
α_2	4×10^8	3×10^8
β_1	3.4×10^8	4.4×10^8
β_2	4×10^8	2×10^8
β_3	4×10^8	3×10^8
γ_1	2.6×10^8	2×10^8
γ_2	4×10^8	1.5×10^7
γ_3	3.5×10^8	1.5×10^8
γ_4	3.5×10^8	4×10^8
γ_5	0.9×10^8	2×10^6

Antiviral Activities of the Various Interferon Species - All human leukocyte interferon species were found to have antiviral activity when assayed with bovine cells (MDBK).[53] The specific activities ranged from 1×10^8 to 4×10^8 units/mg of protein based on bovine serum albumin as a protein weight standard. Measurement of protein by amino acid analysis gave specific activities that were lower by a factor of two. A significant variability among the species was observed when the biological activity was measured on human fibroblasts. The species γ_3 and γ_5 had the lowest specific activity, whereas all other species had a higher biological activity which was similar to that measured with MDBK cells. The specific activities as determined on MDBK and AG-1732 cells are summarized in Table II.

Amino Acid and Amino Sugar Analysis - All the interferon species show significant similarities in their amino acid composition.[53] Some differences between the natural species may reflect extent of glycosylation. However, the amino sugar content of each species analyzed (α_2, β_1, β_2, β_3, and γ_3) was determined to be much less than one residue of either glucosamine or galactosamine per molecule of interferon.[53] We therefore conclude that, contrary to general dogma, human leukocyte interferon is largely devoid of carbohydrate. Nevertheless, we would expect that some species exhibiting molecular weights significantly in excess of 19,000 would contain carbohydrate. In general, these high molecular weight species represent a small fraction of the natural human leukocyte interferons.

The amino-terminal sequence of the first 22 amino acids of leukocyte interferon species IFL-β_1 was determined.[54] The sequence was consistent with that reported for a species of lymphoblastoid interferon with differences in only two positions.[55] These sequence differences confirm the heterogeneity of the species of leukocyte interferon.

HUMAN FIBROBLAST INTERFERON

Several laboratories reported the purification and partial structural analysis of human fibroblast interferon (IFF) with the use of SDS-polyacrylamide gel electrophoresis as the last step in the purification.[30,56] To obtain a product salt- and solvent-free, we developed a

simple two-step purification of fibroblast interferon.[57,58] The first step in the purification from the crude interferon-containing medium involved Blue-Sepharose chromatography, a procedure described previously.[51,56] The second step involved high-performance liquid chromatography on octyl silica. The amino acid composition and 19 residues of the amino-terminal sequence of human fibroblast interferon were determined.[58] The sequence was identical to the first 13 residues reported by Knight et al.[56] and Okamura et al.[59], and the first ten residues reported by Friesen et al.[60] The specific activity of the purified protein is 3 x 10^8 units/mg of protein, similar to that of purified human leukocyte interferon species. Comparison of the first 19 amino acids of human fibroblast interferon with mouse interferons A and B[61] reveals four identical amino acids at positions 3, 6, 11, and 18. It is therefore likely that these proteins are related and have a common ancestral origin.

CLONING AND EXPRESSION OF HUMAN INTERFERONS IN BACTERIA

Although a great deal of research has been directed toward an understanding of the molecular mechanism of interferon induction and activity, progress has been slow due largely to the small amounts of pure interferon protein and mRNA for study. Recombinant DNA technology offers an opportunity to produce a large amount of both the interferon proteins and the interferon gene sequences for use in studying many of these questions.

Recombinants were prepared from mRNA isolated from human leukocytes synthesizing interferon. From these cells, two recombinants were identified: plasmid 104 contained most of the coding sequence for human leukocyte interferon and plasmid 101 contained the sequence for human fibroblast interferon.[62] Comparison of the restriction map of the 104 insert with the data published by Nagata et al.[63] indicated that the plasmid 104 insert has one additional BglII site. Other differences as well are evident in the restriction map. Because plasmid 104 contains more than 80% of the sequence for human leukocyte interferon, this DNA was used as a probe for finding a full-length copy of the interferon cDNA sequence for expression of human leukocyte interferon in Escherichia coli (see below). In addition, this DNA was used to isolate human genomic DNA sequences of the human leukocyte interferons. So far, we have isolated at least ten distinct human genomic sequences[64] for human leukocyte interferon from a human embryonic DNA library in phage λ.[65] With the human fibroblast cDNA, a single genomic clone from the λ library was found corresponding to human fibroblast interferon.[64] So far, none of these genomic clones was found to contain an intervening sequence. These and our previous results demonstrate that leukocyte interferon represents a class of homologous proteins. Furthermore, since we have discovered a number of distinct genomic fragments containing the interferon sequences, it appears that the diversity in human leukocyte interferon is a result of distinct genes representing each expressed human leukocyte interferon sequence.[64,66-69]

Expression of Human Leukocyte Interferon - As noted above, plasmid 104 contained most of the sequence for a human leukocyte interferon, but did not contain the coding sequence for the amino terminus of natural human leukocyte interferon.[62] Accordingly, plasmid 104 was used to screen additional cDNA recombinants. Plasmid (pIFLrA) was identified with sufficient size to code for the entire human leukocyte interferon protein.[68] The 165 amino acid polypeptide constituting the mature human

leukocyte interferon (IFLrA) has a calculated molecular weight of 19,241. Comparing this sequence with other recombinants obtained in our laboratories and elsewhere, we can make the following conclusions. This predicted sequence differs significantly from the complete amino acid sequence deduced from the nucleotide sequence by Mantei et al.[69] In that amino acid sequence, a mature interferon of 166 amino acids is predicted (as in most of our other recombinant clones) that is different from IFLrA which contains 165 amino acids. The extra amino acid (aspartic acid) is between amino acids 43 and 44 of IFLrA. Furthermore, only 137 of the remaining 165 amino acids (83%) are identical.

The mature leukocyte interferon IFLrA was expressed directly by reconstruction of the recombinant.[68] The leader sequence was removed and an ATG-translational initiation codon was placed immediately preceding the codon for amino acid 1 (cysteine) of the mature leukocyte interferon IFLrA. Next, a 300-base pair EcoRI fragment was constructed which contains the E. coli-trp regulatory region. This DNA fragment was then attached to the reconstructed human leukocyte interferon preceding the ATG codon. Clone IFLrA-25 in which the trp promoter was inserted in the desired orientation, yielded high levels of activity: about 2×10^8 units of interferon per liter of culture. The IFLrA protein produced in E. coli behaves similar to authentic human leukocyte interferon in that it is stable to treatment at pH 2 and is neutralized by antiserum to human leukocyte interferon. Furthermore, the IFLrA protein binds to monoclonal antibodies specific for human leukocyte interferon. The IFLrA has been purified to homogeneity with the use of monoclonal antibodies.[70]

Partially purified IFLrA was utilized in an experiment in vivo. It was shown that IFLrA treatment of monkeys prior to infection with encephalomyocarditis virus could prevent the viral disease in squirrel monkeys.[68] These results have demonstrated that the bacterial product is effective in intact animals as well as in tissue cultures. The results confirm our previous conclusions[8,15] that the carbohydrate is not necessary for activity of the interferons. Currently, purified IFLrA is being utilized in human clinical trials.

Synthesis and Expression of Human Fibroblast Interferon in E. coli - Similar to the studies above with human leukocyte interferon, a bacterial clone containing fibroblast interferon cDNA sequences was identified [71] Expression plasmids were constructed which permitted the synthesis of human fibroblast interferon. The fibroblast interferon produced in bacteria is indistinguishable from authentic human fibroblast interferon by several criteria. The amino acid sequence of human fibroblast interferon deduced from the DNA sequence of the full recombinant is identical to that deduced previously from the sequence of cDNA clones.[72-75] Recent experiments indicate that there is only a single human fibroblast interferon gene corresponding to this recombinant.[64,67]

To express mature human fibroblast interferon (IFF) directly in E. coli, a series of plasmids which placed the synthesis of the 166 amino acid polypeptide under trp promoter control were constructed.[71] Human IFF produced by E. coli harboring these expression plasmids behaves like authentic IFF when its antiviral activity is compared on human and bovine cells. It is also stable to treatment at pH 2 and is not neutralized by rabbit anti-human leukocyte interferon antibodies. The amount of human fibroblast interferon synthesized by E. coli was about 8×10^7

units/liter of culture. This corresponds to about 200 micrograms of IFF per liter if a specific activity of 4×10^8 units/mg is assumed.

MONOCLONAL ANTIBODIES TO HUMAN LEUKOCYTE INTERFERON

Monoclonal antibodes have proven to be invaluable for characterization, quantitative analysis, and purification of macromolecular antigens. Of particular interest and value are antibodies against biologically active compounds such as interferon. Until recently, quantitation of interferon could only be determined by relatively complex and time-consuming assays. In addition, purification of the interferons by specific monoclonal antibodies may simplify the purification procedures immensely. Our success in the purification of the human leukocyte interferons made it feasible to attempt the production of interferon-specific monoclonal antibodies by the hybridoma technique of Köhler and Milstein.[76]

Thirteen monoclonal antibodies to human leukocyte interferon were obtained.[77] They exhibit different patterns of binding to purified leukocyte interferon species that are consistent with the structural multiplicity of the human leukocyte interferons. These antibodies have been useful as probes into the structure of the human leukocyte interferons, as well as for their purification and for rapid assay of leukocyte interferon. Thus, these monoclonal antibodies are not only of scientific interest, but also valuable reagents for practical purposes. The majority have been produced and purified in large quantities from ascitic fluid. Most of them are excellent for the affinity purification of the leukocyte interferons produced by either human cell culture or by bacteria expressing cloned interferon sequences. In fact, the monoclonal antibodies have been used to purify human leukocyte interferon expressed in bacteria to homogeneity.[70] In addition, some have been used for the rapid and quantitative determination of interferon with very high sensitivity by radio- and enzyme-immunoassays. In these assays, they did not crossreact with human fibroblast or immune interferons.[78] The relative quantitative binding of the monoclonal antibodies to six different purified interferons indicate that purified leukocyte interferons show structural (antigenic) differences that reflect the multiple human leukocyte interferon genes.

BIOLOGICAL PROPERTIES OF HUMAN INTERFERONS

Numerous cellular-regulatory properties have been attributed to interferon. The ability to inhibit viral growth was the first recognized biological activity of interferon and was the property for which the protein was named. Later, the ability to inhibit cell multiplication, i.e., the antiproliferative activity,[79,80] was detected, as well as immunomodulatory activities.[81] Effective administration of interferon as a therapeutic agent requires a thorough knowledge of its biological properties. The most direct method of obtaining this information involves testing each of the potential biological activities separately with homogeneous interferon. With the availability of pure human leukocyte interferons from both natural sources, as well as from bacteria expressing the human interferons, we have been able to examine several of the biological activities attributed to interferon. We have found that the growth-inhibitory activity of leukocyte interferon purifies with the antiviral activity.[82,83] In addition, we have found the enhancement of natural killer-cell activity is a property of homogeneous leuko-

cyte interferon.[84] Furthermore, homogeneous human leukocyte interferon exhibits inhibition of hemopoietic colony formation on human bone marrow cells.[85]

The antiproliferative effects of purified human leukocyte interferons were measured by their ability to inhibit cell multiplication of the human lymphoblastoid Daudi cell line. The growth-inhibitory and antiviral activities fractionate together throughout the purification of leukocyte interferon. In all cases, those fractions which exhibited antiviral activity also inhibited proliferation of Daudi cells.[82,83] The final stage of protein purification, high performance liquid chromatography on octyl silica particles, pH 4.0, separated at least eight species of leukocyte interferon, all of which possessed growth-inhibitory activity. Because these two biological activities purify together and because the homogeneous species exhibit both activities, we concluded that the antiproliferative and the antiviral activities are both intrinsic properties of these human leukocyte interferons. However, the ratio of the growth-inhibitory to antiviral activity was not constant among the species. In addition, the ratio of the antiviral activity on human cells to that on bovine cells varied from species to species. Similar results were found with interferons purified from a myelocytic cell line.

Whereas, it has not been possible to establish a quantitative correlation between growth-inhibitory and antiviral activity on the basis of these studies with the natural interferons, the results indicated that certain species of interferon may possess a relatively greater potential for inhibiting cell proliferation than others. It is also noteworthy that some fractions that exhibited antiproliferative activity were essentially devoid of antiviral activity. Whether in fact these purified proteins will be considered interferons will require further study.

In addition, since we have expressed a human leukocyte interferon in bacteria, the antiproliferative and antiviral activity of this interferon, IFLrA, was examined. We observed that crude human leukocyte and fibroblast interferon, a purified natural human leukocyte interferon species (IFL-γ_2), purified fibroblast interferon (M. Evinger and S. Pestka, unpublished data), and the recombinant human leukocyte interferon (IFLrA) produced inhibition of Daudi cell growth in essentially equivalent amounts (Fig. 2).[83,86] Thus, we conclude that crude, homogeneous IFL-γ_2, and the recombinant human leukocyte interferon IFLrA, exhibit comparable antiproliferative activity on Daudi cells.

These studies as well as the considerations in preceding sections allow us to conclude that the individual members of the family of human leukocyte interferon proteins differ in structure and in their biological activities. The structural differences are responsible for differences in activity. Since structural information of these interferons is becoming rapidly available from both study of the protein structure itself as well as from the recombinants that have been cloned, a comparison of their structures and activities should provide insight into the primary sequences responsible for these activities. Such information will provide the basic insight for synthesizing active fragments exhibiting all or some of these actions. Our results indicate that optimal molecules for maximal antiviral and antitumor effects may be different so that it will be necessary to evaluate each of the natural and recombi-

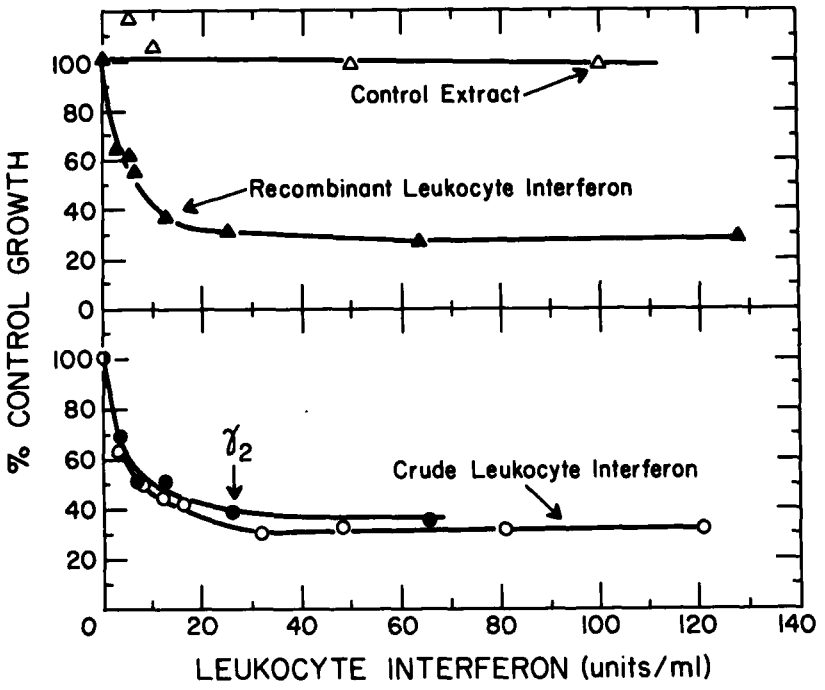

FIGURE 2. Comparison of the growth-inhibitory activity of recombinant leukocyte interferon with that of crude and purified leukocyte interferons. The growth-inhibitory activity of leukocyte interferon was measured with Daudi cells. The amount of interferon added per well is indicated on the abscissa as the antiviral titer in reference units/ml determined on bovine MDBK cells. Control extract was prepared from equivalent numbers of E. coli-carrying nonrecombinant plasmid pBR322 (Δ——Δ). Recombinant leukocyte interferon (IFLrA) is represented by ▲——▲. Crude and homogeneous leukocyte interferon (IFL-γ_2) are represented by 0——0 and ●——●, respectively. (Taken from reference 86.)

nant leukocyte interferons in this regard. Furthermore, it should be possible to modulate selectively these functions by appropriate alterations in their structures.

Since the individual pure leukocyte interferon species exhibit significant variation in biological activities, their evolution and natural functions will be of great interest. Additionally, the modulation of their various activities by structural modifications may have great therapeutic importance, particularly if the number of the existing naturally occurring leukocyte interferon species are augmented by novel species containing modifications and/or new amino acid sequences. Recombinant DNA technology provides a unique and efficient method of generating such novel sequences in large amounts.

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Chapter 22. Monoclonal Antibodies

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Introduction - Monoclonal antibodies have been the dream of many immunologists and biochemists for a long time. The dream was compromised by the reality that a given animal responds to several determinants on one antigen with the production of several antibody species, which vary qualitatively and quantitatively from animal to animal and even with different bleedings of the same animal. Lymphocyte hybridoma technology has made this dream reality. Kohler and Milstein[1,2] ushered in a new era in experimental, clinical and therapeutic research when, by the method of somatic cell hybridization, they successfully generated a continuous "hybridoma" cell line making monoclonal antibody of a defined specificity. They fused a cultured mouse myeloma cell line with splenic lymphocytes from a mouse immunized with sheep red blood cells. These antibody-producing hybrid cells showed characteristics of myeloma cells as they grew continuously in culture, or as solid or ascites tumors in syngeneic mice. In principle, it is possible to obtain an inexhaustible supply of antibody specific for almost any immunogen capable of stimulating B lymphocytes to antibody forming cells. The exquisite specificity of the monoclonal antibodies produced by hybridomas provides possibilities limited only by imagination. Through the use of these monoclonal antibodies, any molecule including histocompatibility antigens, developmental and differentiation antigens, tumor specific antigens, serum proteins, hormones, neurotransmitters and various kinds of receptors can be recognized, isolated, purified, quantified and biochemically characterized.

Following this original discovery a number of investigators have now successfully adapted this methodology to obtain T-lymphocyte hybrid cell lines by fusion of activated T (thymus-derived) lymphocytes with a T lymphoma cell line thereby leading to immortalization of a specific differentiated function.[3-5] These hybrids produce homogeneous effector molecules with a wide variety of immune functions such as enhancement or suppression of antibody responses, generation of helper T cells, suppressor T cells, and cytotoxic T cells. Study of these regulatory molecules will provide greater insight into the genetic, biochemical and molecular mechanisms responsible for cellular development, and the interaction and triggering of various cell types.

The number of published papers on monoclonal antibodies is increasing at an overwhelming pace. This paper covers the progress made in the production of monoclonal antibodies and their role in the study of 1) antibody diversity, 2) the study of cellular differentiation, 3) the detection and analysis of new gene products, 4) the identification of antigens of bacteria, viruses and parasites, 5) practical applications of these antibodies in protection against parasitic and viral infections, 6) general uses of monoclonal antibodies and 7) their anticipated future uses in clinical medicine.

Technique of Making Antibody Producing Hybridomas[6-8] - After appropriate

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stimulation of antibody producing lymphocytes (B lymphocytes) with antigen, usually *in vivo*, these cells are mixed with drug sensitive tumor cells derived from a myeloma in the presence of the fusing agent polyethylene glycol. The fused cells, hereafter called hybrids, are allowed to grow *in vitro* in a controlled tissue culture environment. Use of a selection medium containing hypoxanthine, thymidine and aminopterin (HAT)[9] allows the growth of hydrid cells (hypoxanthine phosphoribosyl transferase$^+$, designated HPRT$^+$), while the tumor cells die because they are HPRT$^-$ and therefore cannot utilize exogenous hypoxanthine and thymidine to synthesize their DNA. In the hybrid cell this enzyme is provided by the parental B lymphocyte. The normal spleen cells are not killed by HAT medium, but they do not grow well in tissue culture and die out or are rapidly outgrown by the hybrids, which double every 17-48 hours. The hybrid cells obtain their ability to grow indefinitely from tumor cells and to make antibody from the stimulated antibody producing B lymphocytes. Therefore the specific antibody production of a normal B lymphocyte has been made immortal through hybridization with a tumor cell and this hybrid cell can be amplified clonally either in tissue culture or by injecting it into an animal to produce a myeloma tumor that secretes large amounts of homogeneous antibody of well defined specificity into the serum or ascites of the tumor-bearing animal. At present, investigators are using nonsecretor parental meyloma cell lines for cell fusion so that the hybrid cells make antibodies of only stimulated B-cell origin. The best hybridoma produce up to 100 μg/ml antibody in tissue culture and 10 mg/ml in ascites fluid.

Production of Human-Human Hybridomas - Several investigators[10,11] have demonstrated that human antibodies of predetermined specificity can be produced by continuously proliferating cell lines. These lines have been established as a result of infection of peripheral blood lymphocytes with Epstein-Barr virus (EBV). This approach has proved to be of limited potential because all EBV-transformed cell lines have been known to lose antibody production with time.

Production of rodent monoclonal antibodies against a wide variety of antigens has been the subject of recent reviews.[12,13] These antibodies have little application for human immunotherapy. For therapeutic purposes antibodies derived from human B lymphocytes are desirable to eliminate the risk of serum sickness, anaphylactic reaction or other allergic sequelae often associated with the administration of non-human proteins to humans. Attempts have been made to immortalize antibody-producing human cells by fusing them with mouse or rat myeloma cells. So far results have been disappointing. These intraspecific hybridomas tend to be unstable, because there is a rapid preferential loss of human chromosomes.[14,15] The availability of appropriate human myeloma lines can facilitate production of human hybridoma products, since in these human-human hybrids repression of human chromosome function or loss of human chromosomes is minimal. Therefore, intensified research efforts have been made to obtain a drug sensitive human myeloma cell line capable of fusing with human B lymphocytes and two laboratories have been successful in this endeavor. Olsson and Kaplan[16] have reported establishment of human-human hybridomas producing monoclonal antibodies against hapten dinitrophenol by fusion of U-266AR$_1$ human myeloma cells with human spleen cells from patients with untreated Hodgkin's disease who had been sensitized to the chemical allergen 2,4-dinitrophenol. Croce *et al.*[17] used an HPRT$^-$ human B-cell line derived from a multiple myeloma patient to fuse with peripheral blood lymphocytes obtained from a patient with subacute sclerosing panencephalitis. Out of the six hybridomas generated, two were found to secrete

human IgM antibodies specific for measles virus nucleocapsid polypeptide. This study shows that it is possible to obtain human-human hybrid cell lines which continuously secrete human antibodies specific for a human pathogenic virus.

1) Monoclonal Antibodies and the Study of Antibody Diversity - The mechanisms responsible for generation of antibody diversity by which lymphocytes become somatically diversified and committed to the expression of a single immunological specificity have been the subject of extensive study and discussion for years.[18,19] Efforts to raise homogeneous antibodies by conventional immunization protocols in amounts to allow structural characterization have met with only partial success.[20-22] Recently hybridoma antibodies have been used to characterize better, previously studied systems based on idiotypic and amino acid sequence data obtained with myeloma proteins.[23,24] Monoclonal antibodies directed against simple antigenic determinants, namely α (1-3) dextran,[25] p-azophenylarsonate[26], phosphorylcholine[27,28], NP(4-hydroxy-3-nitrophenyl) acetyl,[29] and the synthetic terpolymer of glutamic acid, alanine and tyrosine (GAT)[30] have been used to dissect the murine antibody B cell repertoire into individual clonotypes. Idiotypic (variable-region) and amino acid sequence analyses of heavy and light chains of these monoclonal antibodies have provided invaluable information in delineating the mechanisms responsible for antibody diversity. On the basis of amino acid sequence analysis of variable region of heavy chains of monoclonal antibodies directed against α(1-3) dextran, two mechanisms have been proposed for the generation of antibody diversity: a) combinatorial association of multiple V_H (variable region of heavy chain) with multiple J_H (joining region) gene segments and b) additional diversity between the V and J segments from possibly a third variable-region gene segment. Very recently Gearheart et al.[31] with their studies on the amino-acid sequences of several monoclonal antibodies specific for hapten phosphorylcholine have observed that all the proteins with T15 (IgA mouse myeloma protein that binds phosphorylcholine) idiotype are derived from the two identical germ-line genes, but with numerous substitutions, notably in the proteins of ontogenetically later classes, i.e., IgG and IgA. These substitutions can be explained by mutations which appear to be clustered at certain segments of the variable region called hypervariable regions. Hybridomas provide the only way by which one can obtain antibodies which are represented in the antibody repertoire at a very low frequency. Dennis et al.[32] have shown that progeny of a single neonatal virgin B lymphocyte appropriately stimulated with in vitro antigen can be fused with a myeloma cell line to get hybridoma cell lines making homogeneous antibodies. Amino acid sequence analyses of these antibodies should determine the extent of germ line antibody diversity at the neonatal B-cell level. Once this is known it will be easier to understand the mechanisms governing the B-cell repertoire of adult individuals. Hybridoma cells with differentiated functions provide an invaluable starting material to allow a degree of sophisticated molecular dissection at the DNA level.

2) Monoclonal Antibodies as Probes in the Study of Cellular Differentiation - Since its introduction, hybridoma technology has been very successful in generating many new reagents to define markers of cell populations in mouse, rat and humans. Following hybridization to appropriate cell lines, the screening of individual clones and their specific selection are critical steps in identifying those clones that produce antibodies with specificity to differentiation antigens, genetically defined polymorphic antigens, tumor antigens, and functionally important cell-surface molecules.

a) Characterization of Rat, Mouse and Guinea Pig Differentiation Antigens - Rat-mouse and mouse-mouse monoclonal antibodies have been used to study the structure, function and tissue distribution of the antigens encoded in the major histocompatibility complex and characterization of lymphoid cell surface antigens.[33-39] The rat-mouse immunization procedure has the potential of generating hybridomas which detect both polymorphic and non-polymorphic antigens.[39] Target antigens identified by these antibodies have been characterized with respect to charge, size and subunit structure by detergent solubilization, immunoprecipitation and two-dimensional gel analysis. Ledbetter et al.[34] have identified previously known T-cell differentiation antigens Thy-1, Lyt-1 and Lyt-2 plus two new T-cell differentiation antigens. Murine monoclonal antibodies have been made to H-2K, H-2D, I-[35,36], H-2Ld[37], TLA-, and Qa-region antigens[36,38] and to a wide variety of other antigens encoded at various points in and around the major histocompatibility complex. Monclonal antibodies have been used to study sperm cell differentiation in mice[41] and guinea pigs.[41] In the guinea pig study, four monoclonal antibodies recognized differentiation antigens which are restricted to particular regions of the sperm surface.

Springer[42] has used cascade approach for generation of monoclonal antibodies against murine macrophage cell surface antigens of successively lower immunodominance. First monoclonal antibodies against dominant antigens are prepared by using whole cells or antigen prepared from whole cells as the immunogen. These monoclonal antibodies are used to make immunoaffinity columns. Thereafter, detergent solubilized glycoprotein antigens after purification over lectin columns are passed over antibody affinity columns to remove broadly distributed antigens. The antigens not retained by the immunoaffinity column are then used to generate hybridomas making monoclonal antibodies against these antigens. This method of generating monoclonal antibodies can allow identification of further novel cell surface antigens and provides a means to have a complete set of monoclonal antibodies directed against differentiation and developmental antigens and can be used successfully for monoclonal antibody analysis of complex multiple-component biological systems.

b) Characterization of Human T and B-Cell Populations - A number of investigators have made monoclonal antibodies against subsets of human T[43-52] and B lymphocytes.[53-58] Reinherz et al.[43-46] have performed elegant studies with monoclonal antibodies in identification and characterization of cell surface antigens of human thymocytes and T cells. A series of monoclonal antibodies designated as anti-T1, -T3, -T4, -T5, -T6, -T8, -T9 and -T10 have been found to react with human thymocyte and/or peripheral blood T lymphocyte cell surface antigens. Reactivity of most human thymocytes with several of these monoclonal antibodies has suggested that a sizeable portion of thymocytes bear multiple antigens. Detailed studies have shown that human thymocytes consist of three major subpopulations representing different stages of intrathymic differentiation. These subpopulations consist of early thymocytes ($T9^+$,$T10^+$), common thymocytes ($T4^+$,$T5^+$,$T6^+$,$T8^+$,$T10^+$) and mature thymocytes ($T1^+$,$T3^+$,$T5^+$,$T8^+$, $T10^+$ or $T1^+$,$T3^+$,$T4^+$,$T10^+$). It has been shown that aberrations of these regulatory populations exist in a variety of diseases including multiple sclerosis, systemic lupus erythematosus, juvenile rheumatoid arthritis, and infectious diseases such as infectious mononucleosis and lepromatous leprosy. All these cell surface antigens have been extremely useful markers in differentiating between types of thymus-derived acute lymphoblastic leukemias. At least seven different patterns of reactivity with the anti-T-cell antibodies have been found for T-cell acute lymphatic leukemias. T6, T9 and T10 antigens have been shown to be glycoproteins

and preliminary experimental evidence suggests that T6 may be human homolog of the murine thymus leukemia antigen TL.[47]

Several of these monoclonal antibodies against cell surface antigens of human T cells have been well characterized[48] and are now commercially available. Recently an interesting observation has been made with antibody OKT3 (anti-T3)[49]. This hybridoma antibody unlike other monoclonal antibodies currently available inhibits the target cell lysis mediated by allogeneic cytotoxic T cells and generation of these effector cells in mixed lymphocyte culture. This suggests the possibility that monoclonal antibodies with these characteristics have practical applications in clinical immunosuppression and for large scale bone marrow transplantation to deplete donor bone marrow of T cells prior to transplantation in order to reduce risk of graft versus host reaction.

Monoclonal antibodies specific for human T-cells have been used to show T-cell infiltration in the tumors of untreated cancer patients.[50] A monoclonal antibody detecting an antigen on 95-100% of human peripheral blood T lymphocytes, the majority of thymocytes, and acute lymphocytic leukemia T cells but not B cells, B-cell lines or monocytes has been reported by Engleman et al.[51] The glycoprotein antigen identified by this antibody continues to be expressed under various culture conditions with T-cell stimuli as lectin mitogens, soluble antigens and alloantigens. Hence it is a stable T-cell marker. Recently murine monoclonal antibodies have been produced which can distinguish between human cytotoxic T lymphocytes and natural killer (NK) and NK-like cells.[52] Use of these monoclonal antibodies makes it possible to delineate the effector cells that are cytotoxic for a variety of cell lines and autologous malignant cells.

Monoclonal antibody to a human leukocyte specific membrane glycoprotein has facilitated the identification and characterization of the human homologue of the rodent leukocyte common (LC) antigen, a major lentil-lectin binding glycoprotein.[53] This human LC antigen is found on T and B lymphocytes, thymocytes, monocytes, and granulocytes, but absent in other tissues. Recently Dalchau and Fabre[54] have made a monoclonal antibody which reacts strongly with B lymphocytes, only weakly with a small subpopulation of thymocytes and peripheral T lymphocytes and shows no reactivity at all with granulocytes. Stashenko et al.[55,56] have characterized human B lymphocyte-specific surface antigens B_1 and B_2, which are biochemically distinct and are present at different stages of differentiation of human B cells. These antigens are distinct from other B-cell markers including surface immunoglobulin, Ia like antigens, and Fc and C3 receptors. Preliminary results indicate that B_2 antigen may be clinically useful since it is present on B cell-derived non-Hodgkin's lymphomas and the majority of non-T cell acute lymphoblastic leukemias that express the common antigen. Kersey et al.[57] have made a monoclonal antibody against a pre-B form of acute lymphoblastic leukemia which is present on the surface of lymphoid leukemic cells, bone marrow lymphohemopoietic progenitor cells and most of non-T and non-B acute lymphoblastic leukemia. A monoclonal antibody made against a human B lymphoblastoid cell line has been shown to react with some of the B-cell lines and the B-enriched fraction from peripheral blood mononuclear cells but not with T- and null cell lines.[58] Monoclonal antibodies recognizing all or certain subpopulations of human monocytes have been reported.[59]

c) Monoclonal Antibodies Against Tumor Associated Antigens - There is considerable argument over whether or not tumor-specific antigens exist in man. Studies with monoclonal antibodies can resolve this question. Two groups have reported production of monoclonal antibodies that react with human melanomas[60,61] from several individuals and not with non-malignant cells from the same patient whose melanoma cells were used to immunize mice or with other types of human cells including tumors of other tissue origins. It was also shown that nude mice carrying cells of the anti-melanoma hybridoma destroyed an injected human melanoma cell line.[60] Henlyn et al.[62] have made monoclonal antibodies which react with human colorectal carcinoma cells and not with normal colonic mucosa or other normal and malignant human cells. Kennet and Gilbert[63] have characterized a monoclonal antibody against human neuroblastoma. This antibody detects the antigen on fetal brain and neuroblastoma and does not react with adult brain, fetal tissues other than brain or lymphoblastoid cell lines. Monoclonal antibodies produced against glycoprotein antigens[64,65] of mammary tumor viruses of laboratory mice have facilitated the detection of distinct antigenic determinants which represent type, group, and interspecies reactivities.

In understanding the development of neoplastic disease monoclonal antibodies can be used to study a) antigenic determinants identical for a given tumor cell and/or cross reacting with other human cells, b) fine specificities of antigens present on tumor cells and normal cells, c) immunological and biochemical changes in antigen expression within a single individual and d) isolation and biochemical characterization of molecules which appear to be tumor specific.

3) Monclonal Antibodies and Study of Human Gene Products

a) Complement System - The complement system, an integral part of the immune system, consists of over 20 immunochemically distinct proteins capable of interacting with each other, with antibody and with cell membranes. Cells of the immune system bear complement specific receptors which are important for their function. Clq is the first subcomponent of the first component (C1) of the classical complement pathway and it binds to antigen/antibody complexes on the cell surface which leads to activation of cascade of reactions resulting in cell lysis. Soloman and Reid[66] have made monoclonal antibody against Clq and showed that this antibody inhibited the activity of human serum Clq.

b) HLA Antigens and β_2- Microglobulin - The major human histocompatibility (MHC) region in man is an extremely complex genetic region coding for eight different loci, all functionally related within the immune system. This region is responsible for multiple immunologic processes, including the immune response to a variety of antigens, the generation and specificity of cytotoxic killer cells, the restrictions in cell-cell interactions and autoimmune reactions.

The HLA-D/DR locus is considered to be the human counterpart of the I subregion of the murine major histocompatibility complex. The Ia (immune response region associated) antigens of the mouse are the basis for the genetic control of the immune response. The HLA-D/DR antigens are polymorphic. Genes coding for the HLA-A, -B and -C polymorphic determinants, HLA-DRw specificities (Ia glycoproteins), the complement components (C2, C4 and factor B) and many other determinants involved in various immune phenomenon are located on chromosome 6. It is apparent that better definition of these gene products can contribute to our understanding of the biologic role of the MHC.

Monoclonal antibodies with specificity for both polymorphic and nonpolymorphic determinants of HLA-ABC glycoproteins,[67,68] anti-β_2-microglobulin[69] and both polymorphic and nonpolymorphic sites of HLA-DRw[70-75] specificities have been raised in inbred mice using papain purified antigens as well as whole cell membranes as immunogens. Monoclonal antibody reagents like these are essential to correlate molecular structure with the functional repertoire of the human Ia-like antigens.[76] Monoclonal antibodies will permit more rigorous studies of the tissue distribution and organ distribution of a variety of antigens controlled by the MHC, the isolation and purification of the HLA-A, B, C and DR antigens, as well as their immunochemical studies of molecular evolution, analysis of *in vitro* systems involving cell-cell interactions, and also the definition of new probes capable of identifying disease associations and susceptibilities.

c) Genetic Analysis of Enzymes - Slaughter *et al.*[77] have successfully applied the hybridoma technique for detection of enzyme polymorphism. Six monoclonal antibodies raised against human alkaline phosphatase were assayed for reactivity pattern against a panel of 295 placental extracts that had previously been typed electrophoretically. By using these antibodies these investigators have been able to detect three common alleles, as well as several rare alleles.

4) Monoclonal Antibodies to Microorganisms - Gerhard *et al.*[78] have used monoclonal antibodies against influenza virus to study (a) the expression of viral proteins on infected cells to determine antigenic drift, hence, select mutants of the influenza virus expressing antigenically altered hemagglutinin (HA) molecules, (b) to construct an operational antigenic map of the HA molecule by comparative antigenic analysis of the mutant viruses, (c) to determine the B-cell clonotype repertoire of anti-hemagglutinin, and (d) to study antibody-mediated blocking of antiviral cytotoxic T-cell activity. Fifty eight monoclonal antibodies against HA have been used to analyze 34 mutant viruses and four antigenic sites on the HA molecule have been defined. These sites have undergone antigenic drift to a different extent in nature which implies that the mechanisms responsible for antigenic drift act selectively on distinct structures of the HA molecule.

Flamand *et al.* have used anti-nucleocapsid[79] and anti-glycoprotein[80] hybridoma monoclonal antibodies to detect the antigenic differences between rabies and rabies-related virus proteins and have found extremely subtle differences among populations of viruses. These subtle differences among rabies viruses and rabies-related viruses have been suggested as a possible explanation of perplexing variations in the effectiveness of rabies vaccine in different parts of the world.

Nowinski *et al.*[81] have mapped the sites of five different epitopes on the viral envelope polypeptide by using monoclonal antibodies against murine leukemia virus. Monoclonal antibodies against EBV[82], Herpes simplex virus[83,84] and lymphocytic choriomeningitis[85] virus have been reported. Antibody against the major glycoprotein of EBV has been shown to neutralize EBV *in vitro* and immunoprecipitate both gp350 and gp220 from the membrane antigen mixture.[86] Niman and Elder[87] have performed detailed studies of molecular dissecton of Rauscher virus gp70 by using hybridomas raised against purified virus gp70. Using a panel of 56 monoclonal antibodies these investigators have defined three domains of the molecule. Homologies and nonhomologies between the recombinant viruses defined by

monoclonal antibodies have been confirmed by peptide fingerprinting of Rauscher-gp70, the recombinant gp70's and their respective breakdown products.

It is anticipated that antimicrobial monoclonal antibodies of predetermined specificity can be successfully used (a) to detect subtle antigenic differences in a given bacteria or virus which are not discernable using conventional antibodies, (b) to study the phenomenon of antigenic drift at the level of individual determinants of a particular viral antigen molecule, (c) for comparative study of the frequency of antigenic mutants among various types of viruses, (d) for accurate mapping of the antigenicity of viral proteins, (e) determination of the relationship among isotype, fine specificity, affinity, and functional properties of antiviral antibodies both *in vitro* and *in vivo*, (f) isolation and characterization of virus variants obtained *in vitro* from clones of the virus grown in tissue culture in the presence of monoclonal antibodies and *in vivo* from animals bearing antibody-producing hybridoma clones and infected with fixed or street virus-strains, (g) biochemical analysis of variants showing individual specificity in binding assays with a panel of monoclonal antibodies, (h) to identify quickly and accurately bacterial isolates in clinical laboratories, and (i) to detect microbial antigens in tissue specimens. Rapid identification of a bacterial pathogen is critical for infections in immunocompromised or critically ill hosts.

5) Studies Involving Practical Applications of Monoclonal Antibodies - Monoclonal antibodies, being standardized reagents, have great potential as diagnostic reagents of great sensitivity. High affinity monoclonal immunoglobulins of IgG and IgM classes have been produced against the hepatitis B surface antigen.[88] These monoclonal antibodies have been shown to detect hepatitis B surface antigen-associated determinant in acute hepatitis and post-transfusion hepatitis.

Use of monoclonal antibodies in targeting cytotoxic drugs to specific tissues has been explored with murine monoclonal antibodies raised against human colorectal carcinoma cells.[89] Cell-specific cytotoxic agents have been made by covalently coupling the A chain from diphtheria toxin or ricin toxin to the monoclonal antibody directed against a colorectal carcinoma tumor-associated antigen. Antibody toxin conjugates *in vitro* were found to be highly specific in killing cells bearing the right antigen but had no cytotoxic effect on cell lines lacking the antigen. Recently Blythman *et al.*[90] have found that murine monoclonal antibodies of the IgM class directed against the Thy 1.2 differentiation antigen of mouse T cells when coupled with ricin-A chain killed mouse leukemia cells carrying the same antigen. Cytotoxic activity of the monoclonal antibody and toxin subunit conjugate, called immunotoxin, was specific as it had no effect against Thy 1.1 cells. These studies carried out *in vivo* prolonged the time of survival in mice treated with immunotoxin. These studies strongly suggest the potential use of immunotoxins as a highly sensitive test system for studies relating to treatment of cancer. In the past, several investigators tried to use antibodies to target the toxic activity of cytotoxic drugs to tumor cells but lack of high-titer antibody against specific cell surface antigens has been a major limiting factor. With these antibodies it is possible to ask the questions concerning which class, subclasses or antibody fragments may be more efficient in promoting the productive internalization of covalently coupled toxin.

Hybridoma antibodies have been used successfully to enhance renal allografts in rats.[91] This is the first in vivo demonstration whereby passively transferred alloantibodies against major histocompatibility determinants were fully capable of inducing a state of immunological enhancement. Recently, Cosimi et al.[92] have shown that monoclonal antibody to OKT4, an antigen on T inducer cells, had an immunosuppressive effect when given to monkeys and resulted in prolonged kidney-graft survival. Results obtained with conventional antisera have suggested a potential role for antibody therapy, but the success of this approach has been difficult to assess because of the limited quantities of high-titer antibody of appropriate class, affinity and specificity. Bernstein et al.[93] have used a monoclonal antibody against a normal differentiation antigen (Thy-1) for the treatment of murine leukemia of spontaneous origin. A monoclonal antibody to rabies virus[94] has been shown to be protective in mice against infection with lethal doses of rabies virus.

Use of Monoclonal Antibodies as Protective Agents Against Parasites - Possible roles for monoclonal antibodies in direct therapy against parasites are under serious consideration. In this respect two kinds of roles can be envisioned for monoclonal antibodies: firstly, anti-parasite monoclonal antibody-cytoxic drug conjugates can be used to carry the drug to the target parasite, thus concentrating the drug's effect. Secondly, it is feasible to produce monoclonal antibodies against parasite antigens which will themselves find and attach the invading parasites, thus intervening in the life cycle of the parasitic organism.

Monoclonal antibodies potential in the war against malaria has been shown by two research groups.[95,96] These investigators raised monoclonal antibodies to surface antigens of Plasmodium berghei, which interfered with rodent malaria infection at the sporozoite or merozoite stage. One monoclonal antibody was of the IgG1 subclass directed against a protein antigen (Pb44) that is present on the surface membranes of sporozoites. This antibody abolished the infectivity of the parasites both in vivo and in vitro studies. Recently, Perrin et al.[97] have reported that a monoclonal antibody against Plasmodium falciparum can inhibit infection by this parasite in cultures of human erythrocytes. An extensive study of a spectrum of monoclonal antibodies against P. falciparum will help to define antigens required for protection and effective mechanisms of immunity. With the use of these monoclonal antibodies, it will be possible to purify potentially protective antigens of P. berghi and P. falciparum and other parasites as well. Because obtaining large quantities of Plasmodium antigens is not practical, one can utilize the DNA recombinant technology to produce defined malarial antigens. In this respect, monoclonal antibodies will be extremely useful for identification of bacterial colonies secreting the desired antigens. Monoclonal antibodies specific for schistosomal antigens[98,99], Theileria parva[100] and Toxoplasma gondii[101] parasitic antigens which can be used diagnostically, have been reported.

6) General Uses of Monoclonal Antibodies - Since hybridoma technology in essence clones out of the spleen individual antibody-forming cells, it provides the only way by which one can generate homogeneous antibodies against impure antigens. This has been shown in the case of monoclonal antibodies against interferon[102,103] and differentiation antigens on subsets of both human and rodent lymphocytes. Secher and Burke[102] have made monoclonal antibody against interferon by immunizing mice with a crude preparation of antigen which contained only 0.1-1% interferon.

A number of monoclonal antibodies have been generated that are specific for normal cell products or their receptors. Substance P is involved in the pain pathways in the brain. A monoclonal antibody against this peptide has been made[104] and this antibody can be used to study its physiology. Monoclonal antibody has been produced against osteoclast-activating factor.[105] This antibody will be highly useful in determining the nature of this peptide and its quantification in diseases such as multiple myeloma, where tumor deposits are associated with osteolytic lesions. Monoclonal antibody against acetylcholine receptor[106] has been shown to cause myasthenia gravis. This antibody caused this autoimmune syndrome without blocking the receptor function. Monoclonal antibodies against β-adrenergic receptors[107] have been utilized in purification and molecular characterization of these receptors.

As monoclonal antibodies can be made in unlimited supply, they will be greatly used in genetic studies such as mRNA and gene isolation, chromosomal location of specific genes, immunoglobulin structure, dectection of new or rare immunoglobulin gene products, structural studies of enzymes and other proteins and structural and population studies of protein polymorphisms. They may replace conventional antisera for studies of chromosome structure and function, gene mapping, embryogenesis, characterization and biosynthesis of developmental and differentiation antigens specific for various cell types and tissues, species specific antigens, antigens involved in chemotaxis, immunogenetics and clinical genetics, including genetically inherited disorders, chromosome aberrations and transplantation antigens. Hybridoma antibody products provide a unique way of recognizing antigens against which efforts to raise conventional antibodies have been unsuccessful. Because hybridomas can be generated across species, researchers can now identify molecules that do not exist as allelic polymorphisms and are therefore not recognized in alloimmunizations.

7) Anticipated Uses of Monoclonal Antibodies in Medicine - Revolutionary advances in the understanding and treatment of disease, development of vaccines and promotion of organ transplantation appear to be imminent through applications of hybridoma technology. Monoclonal antibodies are being used increasingly in research in cancer, heart disease, multiple sclerosis, viral, bacterial, rickettsial infections, disorders of the immunological defense system, and in many basic studies of the physiology and development of the body in both the normal and diseased state.

Administration of human, rather than xenogeneic, antibodies (anti-lymphocyte globulins, anti-thymocyte globulins, and a variety of antibodies to bacterial viral and other antigens) to patients can eliminate the risk of allergic reactions. As each monoclonal antibody is directed against a single determinant it attains a finer, more specific recognition of its antigen than conventional antibodies. Scientists can use these antibodies as exquisitely sensitive probes to be guided to target cells or organs, thereby aiding in the identification or mapping of these drug targets in the body. They can also be used to deliver cytotoxic drugs to malignant cells or enzymes to specific cell types. A battery of monoclonal antibodies can be used to detect changes in the biology and chemistry of patients during flare-ups of certain human diseases. Antibodies against different subsets of T lymphocytes, particularly suppressor lymphocytes, have been and will continue to be of tremendous value in the diagnosis and treatment of patients with multiple sclerosis, heart diseases and leukemias. As more and more monoclonal antibodies directed against normal and tumor cells are generated, it is possible to have a panel of these antibodies, each of which will define a distinct molecular determinant

on the cell surface to better define the stages of lymphoid cell differentiation, as well as a more precise and reliable classification of cell malignancies and immunodeficiencies in humans.

Monoclonal antibodies will also be explored for their ability to seek out and neutralize either excessive amounts of drugs or toxic drug metabolites in patient's blood. Use of monoclonal antibodies to obtain highly sensitive fingerprinting of viral and mammalian parasitic antigens from different parts of the globe can be extremely useful to develop vaccines for immunization against viral and parasitic diseases. Studies demonstrating the protective properties of monoclonal antibodies against malarial parasite of mice suggest the possibility of doing the same study with human malarial parasites.

Monoclonal antibodies, being standardized pure reagents, are replacing conventionally made tissue-typing reagents. These will undoubtedly help planning more rational therapeutic strategies for treatment and prognosis of certain diseases, because different types of cells may respond to different drugs. Monoclonal antibodies, by themselves or in combination with surgery, have a strong potential for immunotherapy of serum hepatitis, leukemias, etc. Monoclonal antibodies will open up potential new immunodiagnostic markets for identification of various leukemic and lymphoma cell populations, characterization of isoenzymes, hemogloblins, α-1-antitrypsins, lymphokines, hormones, hepatitis-associated antigens, carcinoembryonic antigens and assays for therapeutic drug monitoring, abused drug monitoring and specific protein immunoassays. As well defined chemical reagents, monoclonal antibodies have great potential for practical applications in man either by their direct use or through their utilization in drug development. It is safe to assume that the practical usefulness of human-human hybridomas in man lies in the not too distant future.

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Chapter 23. Calcium Antagonists

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Introduction - Calcium ions appear to play a universal role in physiological body functions. Thus, calcium is involved in blood clotting and coagulation, cellular adhesion and integrity and membrane stability, bone and teeth formation, enzyme activity, control of certain aspects of cyclic nucleotide metabolism, mediation of some actions of prostaglandins, cell division, muscle contraction, glandular and other cellular secretory functions, neuronal transmission, and undoubtedly many more physiological functions. Calcium also mediates the pharmacological and toxicological actions of numerous drugs and chemicals. Thus, drugs, which in most cases have only recently been recognized as calcium antagonists, have enjoyed a wide variety of therapeutic applications as local anesthetics, anticonvulsants, antiarrhythmics, coronary dilators, antihypertensives, and skeletal muscle relaxants among others.[1,2]

The source of calcium for the physiological functions cited above is either the extracellular compartments (the basement membrane, the ground substance, or the extracellular fluids) or the intracellular calcium storage pools (mitochondria, endoplasmic reticulum, nucleus, inner aspect of the plasma membrane, and possibly secretory vesicles).[1-6] Drugs which activate calcium-dependent processes do so by increasing the level of cytoplasmic free calcium by enhancing the influx of extracellular calcium, by mobilizing intracellular calcium, or by preventing sequestration or efflux of cytosolic calcium.[1-3,6] Extracellular calcium entering the cell is frequently referred to as "trigger calcium", whereas the intracellular calcium ultimately directly responsible for the physiological and pharmacological response is frequently referred to as "activator calcium".[7] The extracellular calcium, the intracellular calcium, or both (depending upon the tissue and the stimulus) are involved in initiating the physiological or pharmacological response. In instances where calcium-dependent cells are activated by membrane depolarization (e.g. myocardial cells), depolarization of the cell membrane during the action potential is mediated primarily by two inward currents: an initial fast current carried by extracellular sodium ions entering the cell through the fast sodium channels in the cell membrane, and a subsequent slow current partly carried by extracellular calcium entering the cell through slow calcium channels in the cell membrane.[8] It should be noted, however, that access of extracellular calcium to the intracellular space is not limited to the membrane slow calcium channels.[9]

Calcium Antagonists - Calcium antagonists can be classified into two groups: calcium entry blockers (membrane calcium antagonists), and intracellular calcium antagonists. In most tissues, calcium is highly compartmentalized with ionic activities across cellular and intracellular membranes often differing by a factor of 1000 or more.[10-12] The active

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mechanisms regulating the transport of calcium across membranes are still incompletely understood.

Calcium Entry Blockers - The calcium entry blockers act primarily (but not exclusively) by inhibiting the influx of extracellular calcium into cells through the slow calcium channel in the cell membrane. Such calcium entry blockers include local anesthetics, manganese, lanthanum, phenytoin, barbiturates, compound Org 6001 (3α-amino-2β-hydroxy-5α-androstan-17-one), methadone, ℓ-acetylmethadol, ℓ-pentazocine, dantrolene, nitroglycerin and other nitrites and organic nitrates, indomethacin, adrenergic β_2-receptor agonists, morphine, alcohol, aminoglycoside antibiotics (streptomycin, neomycin), SKF-525A, R33711, flunarizine, cinnarizine, hydralazine, lidoflazine, bepridil, cinepazet maleate, hexoestrol, and the prenylamine group [prenylamine, verapamil, methoxyverapamil (D600), fendiline, nifedipine, diltiazem, perhexiline, FR 7534].[13-36] All of these compounds differ markedly in chemical structure.[10]

Although inhibition of the slow inward calcium current is a property common to all calcium entry blockers, most of these agents possess other major pharmacological properties which most assuredly contribute to their mechanisms of action. Thus, local anesthetics block the rapid inward sodium channel.[37] The coronary dilating nitrites and lidoflazine enhance adenosine activity,[13,38] and the nitrites additionally increase the levels of smooth muscle cyclic AMP.[38] The latter property is also shared by the adrenergic β_2-receptor agonists in their smooth muscle relaxing properties.[38] The antiinflammatory properties of indomethacin correlate well with both its calcium antagonistic action as well as with its ability to inhibit prostaglandin synthesis.[25]

A number of calcium entry blockers of the prenylamine group possess an asymmetrical carbon atom and occur as optical isomers, with each isomer exhibiting different pharmacological properties. Thus, the (+)-isomers of racemic verapamil, prenylamine, and D600 are predominantly fast sodium channel inhibitors, whereas the (-)-isomers are predominantly slow calcium channel blockers.[39,40] Such findings are of particular importance, since the racemic form of these compounds is generally used and the pharmacological observations (coronary dilation, antiarrhythmic action) are invariably attributed solely to calcium antagonism while ignoring the contribution of sodium antagonism. In addition to the inhibition of transmembrane calcium and sodium influx produced by racemic prenylamine, this drug exhibits antiadrenergic effects,[41] including α-receptor[42] and β-receptor[43] blocking properties, and depletion of neuronal norepinephrine and dopamine.[44,45] Nifedipine, probably the most potent of the prenylamine group of calcium entry blockers, appears to block exclusively the slow calcium channel.[40,46] On the other hand, diltiazem has been shown to block the fast sodium channel,[47] in addition to the slow calcium channel. The complexity of the pharmacological and electrophysiological properties of the prenylamine group of calcium antagonists has recently been reviewed.[10]

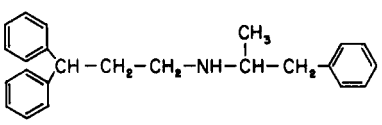
Thus, the original concept, based on electrophysiological voltage clamp experiments and on experiments with glycerinated muscle fibers,[19,48,49] that members of the prenylamine group of compounds do not significantly interfere with the inward fast sodium current but significantly and reversibly block the slow inward calcium current without interfering with the intracellular action or disposition of calcium, requires modification. This is particularly true in light of recent findings indicating that (±)-verapamil rapidly accumulates in cardiac cells,[50] has no effect

on the uptake of ^{45}Ca by isolated atria or on the total tissue calcium content,[50] does not influence the binding of calcium ions by isolated myocardial plasma membrane vesicles,[50] and does not influence calcium movements in isolated ventricular sarcoplasmic reticulum preparations[51,52] nor in mitochondrial preparations.[50] Similar findings with D600[50] support the notion that this group of compounds may not exert their negative inotropic actions by effects on calcium movements at the level of the plasma membrane, sarcoplasmic reticulum, or mitochondria. On the other hand, a direct influence on the contractile system could explain the cardiodepressive actions of verapamil, since it has recently been demonstrated that verapamil delays the ATP-evoked superprecipitation of ventricular myosin-B.[50]

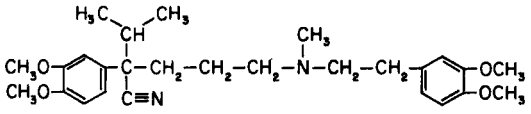
Regardless of the controversy surrounding the molecular mechanism of action of the prenylamine group of drugs, these agents have enjoyed considerable clinical success in the treatment of stress angina pectoris, Prinzmetal variant angina, certain cardiac arrhythmias, and possibly hypertension. This group of drugs offers a number of theoretical and practical advantages over the nitrites and adrenergic β-receptor blockers in the management of angina pectoris.[1,19,53,54] Although it has been argued that the antiarrhythmic effect of the prenylamine group of drugs is restricted to those agents or isomers exhibiting fast sodium channel blocking properties,[10] it has been convincingly demonstrated that (-)-verapamil was at least as potent as (+)-verapamil in preventing ventricular fibrillation in coronary ligated dogs.[55] Since after coronary artery ligation the cells of the injured myocardium may be partially depolarized with a resultant partial or total inactivation of the fast sodium channels, and further depolarization and inhomogenous conduction may result mainly from calcium currents flowing through the slow calcium channels,[55] it follows that (-)-verapamil should be effective in preventing ventricular fibrillation in this setting. The effectiveness of (+)-verapamil in this situation may be attributable to suppression of ventricular ectopic pacemaker activity which is at least partially dependent on the residual fast sodium channel activity.[55] Clinically, (±)-verapamil is effective in the treatment of supraventricular arrhythmias and in the prevention (but not the treatment) of ventricular arrhythmias during the initial stages of a myocardial infarction.[56] On the other hand, nifedipine is devoid of antiarrhythmic activity.[57] Such enigmatic observations await clarification.

Intracellular Calcium Antagonists - Intracellular calcium antagonists interfere with the physiological functions of calcium by inhibiting the action or mobilization of intracellular calcium, or by enhancing the sequestration of this cation by intracellular organelles or its efflux from the cell.[1,2] Pharmacological agents which act, at least in part, as intracellular calcium antagonists include magnesium,[5,58-64] sodium nitroprusside,[65] diazoxide,[65] dantrolene,[66,67] ryanodine,[68,69] the TMB compounds [ω-(N,N-diethylamino)alkyl-3,4,5-trimethoxybenzoates],[70-72] and the MDI compounds (2,3-substituted-5,6-methylenedioxyindenes) developed in our laboratories.[73-78] With the exception of dantrolene (which is a skeletal muscle relaxant), all of these agents are cardiovascular depressant drugs by virtue of their calcium antagonistic properties, and their pharmacology has been recently reviewed.[1]

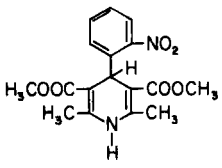
The remainder of this chapter will be devoted to a review of the chemistry,[73,74,79-81] basic[74,75,82] and applied[76-78] pharmacology, and toxicology[83] of the MDI compounds developed and tested in our laboratories, and their comparison with other calcium antagonists. The structures



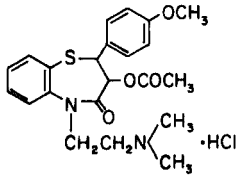
Prenylamine



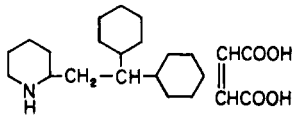
Verapamil



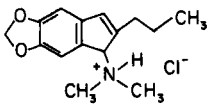
Nifedipine



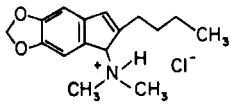
Diltiazem



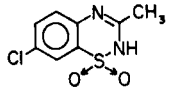
Perhexiline



pr-MDI



bu-MDI



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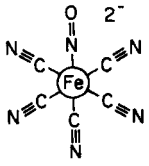

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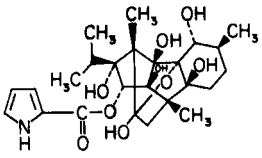

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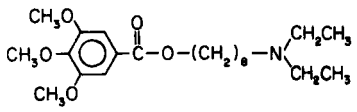

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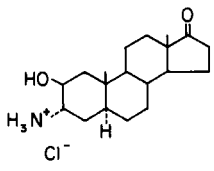

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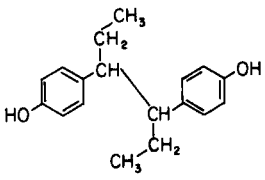

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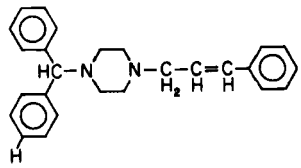

Cinnarazine

Figure 1. Chemical structures of some calcium antagonists.

of 2-n-propyl- and 2-n-butyl-3-dimethylamino-MDI (pr-MDI and bu-MDI, respectively) are shown in Figure 1 along with the structures of some other calcium antagonists. Additional chemical considerations are discussed elsewhere.[84]

Development of the MDIs - The MDIs were obtained as intermediates[73] in the synthesis of potential prostaglandin (PG) antagonistic end products.[80] As a consequence, our original pharmacological experiments were performed on the isolated rat uterus[74] with the intention of screening the intermediate MDIs for potential PG receptor antagonistic activity. However, our early observation that the MDIs were not selective PG blockers led us to suspect that they may be acting by a more general mechanism, such as calcium antagonism.[74,75]

Basic Pharmacological Studies with the MDIs - The pr-MDI and bu-MDI ($5x10^{-5}$ - 10^{-4}M) blocked the spasmogenic action on the estrogenized rat uterus of $PGF_{2\alpha}$, PGE_2, oxytocin, barium, acetylcholine, and ergonovine in a concentration-dependent and reversible manner.[74] Furthermore, the MDIs blocked the contractile effect of histamine on the isolated guinea pig ileum, and of acetylcholine on the isolated rat ileum.[74] The antagonism by the MDIs of the spasmogenic action of acetylcholine (which utilizes extracellular calcium) and barium (which utilizes intracellular calcium) could be reversed by increasing the extracellular calcium concentration,[74] indicating that the MDIs were interfering with excitation-contraction coupling by acting as calcium antagonists with a probable intracellular site of action.

To further characterize the calcium-antagonistic mechanism of action of the MDIs, we utilized the isolated perfused bovine adrenal medulla as a model for stimulus-secretion coupling,[75] since this preparation has been shown to share many common molecular features with excitation-contraction coupling in muscle.[3-5] Adrenal catecholamine secretion induced by carbachol is known to be mediated by extracellular calcium[85] and not by intracellular calcium.[86] On the other hand, acetaldehyde-induced adrenomedullary catecholamine secretion is independent of both extracellular calcium[62,63,87] and intracellular calcium.[62] The pr-MDI and bu-MDI (10^{-8} - 10^{-4}M) reversibly blocked adrenomedullary catecholamine secretion evoked by carbachol, but did not affect acetaldehyde-induced catecholamine secretion.[75] Furthermore, these MDIs did not affect ^{45}Ca uptake by adrenomedullary chromaffin cells.[75] These findings indicated that the MDIs were interfering with stimulus-secretion coupling by acting as calcium antagonists with a probable intracellular site of action.

Further pharmacological confirmation of an intracellular site of action for the MDIs derives from the findings that pr-MDI enhances calcium uptake (sequestration) by isolated myocardial sarcoplasmic reticulum fractions,[88] and bu-MDI inhibits calcium release (mobilization) from intracellular storage sites in skeletal muscle as evidenced by a decrease in activation heat[89] (which represents the energy liberated in association with calcium mobilization and sequestration in contracting muscle). Furthermore, caffeine-induced contractures of skeletal muscle (which are dependent upon intracellular calcium) are blocked by pr-MDI (Rahwan and Gerald, submitted).

Basic Biochemical Studies with the MDIs - In order to further characterize the intracellular mechanism of action of the MDIs, their interaction with intracellular calcium receptors was examined.[82] It is now believed that many of the second messenger effects of calcium are mediated by the

intracellular calcium binding proteins (receptors), calmodulin and troponin-C.[90-92] Equilibrium dialysis showed that bu-MDI bound to calmodulin in a calcium-dependent manner at four equivalent sites of equal affinity (6.2×10^{-4}M), over the range of 10^{-7} to 10^{-5}M free calcium which would be expected to occur physiologically. One-half maximal binding occurred at a free calcium concentration of 7×10^{-6}M. The interaction between bu-MDI and calmodulin was competitive in nature, and bu-MDI was capable of inhibiting a number of calcium-calmodulin-dependent processes.[82] Furthermore, bu-MDI was capable of binding to cardiac troponin-C.[82] Such findings indicate that the MDIs may interfere with excitation-contraction coupling through their interaction with intracellular calcium receptors. Prenylamine has also been shown to interact with calcium-calmodulin and inhibit certain calcium-calmodulin-dependent processes.[122,123]

Basic Electrophysiological Studies with the MDIs - In the case of the prenylamine group of drugs, electrophysiological studies point to a major mechanism of action involving inhibition of calcium entry into cells,[19,48,49] whereas pharmacological studies (with verapamil,[50-52] D600,[50] and diltiazem[93,94]) point to an intracellular locus of action involving inhibition of mobilization (in the case of diltiazem) or action (in the case of verapamil and D600) of intracellular calcium. The same dichotomy between electrophysiological and pharmacological results[74,75,82,89] is also evident with the MDIs. Thus, although the pharmacological and biochemical studies point to an intracellular site for the calcium-antagonistic properties of the MDIs, concentrations of 10^{-6} to 10^{-4}M of pr-MDI or bu-MDI produce electrophysiological changes in canine Purkinje and cardiac ventricular muscle fibers that indicate their ability to interfere with both the fast (sodium-dependent) and the slow (calcium-dependent) components of the cardiac action potential.

In cardiac Purkinje fibers superfused with Tyrode's solution ($[K^+]_o$ = 4mM), concentrations of pr-MDI and bu-MDI greater than 10^{-6}M decreased action potential amplitude, overshoot, maximum rate of rise of phase 0 ($\dot{V}max$) and duration to 50% repolarization. Action potential duration at 90% repolarization was unchanged at 10^{-6} to 5×10^{-5}M MDI concentrations and decreased at drug concentrations greater than 10^{-4}M. Resting membrane potential was unchanged from control. The slopes of phase 1 and 3 of the cardiac action potential decreased, while the slope of phase 2 increased and was shifted to more negative potentials. The effects of superfusion with pr-MDI or bu-MDI on the transmembrane potential of Purkinje fibers appeared dependent upon both drug concentration and stimulation rate. In addition, MDI concentrations in excess of 10^{-6}M depressed membrane responsiveness as indicated by a shift of the curve relating $\dot{V}max$ to membrane potential down and to the right.

Action potentials recorded from ventricular muscle preparations demonstrated changes that were unlike the effects observed in Purkinje fibers. Increases in action potential duration at 50 and 90% repolarization were observed at all MDI concentrations studied (10^{-6} - 2×10^{-4}M). Phase 2 became more prominent and was increased in duration. The slope of phase 3 was decreased and associated with lengthening of action potential duration at 50 and 90% repolarization. The changes in ventricular muscle action potential characteristics were most obvious when bu-MDI was added to the superfusion medium and occurred simultaneously with decreases in $\dot{V}max$ and developed tension.

The pr- and bu-MDIs demonstrated no significant effect on the slope of phase 4 depolarization in Purkinje fibers stimulated every 1000 msec

and superfused in Tyrode's solution ($[K^+]_o$ = 4mM). In driven preparations (cycle length = 1000 msec) superfused with 2 mM K^+ and 10^{-6} to 10^{-5}M pr-MDI or bu-MDI, the slope and magnitude of phase 4 increased initially and then decreased. The activation voltage became less negative. MDI concentrations greater than 10^{-4}M decreased the slope of phase 4 and resulted in total nonresponsiveness to external stimulation. In Purkinje fibers exhibiting spontaneous normal automaticity due to hypokalemia ($[K^+]_o$ = 2mM) or catecholamines (10^{-7}M epinephrine), the MDIs produced an increase in the slope of phase 4 depolarization and reduction in the membrane voltage for activation (10^{-6} to 10^{-5}M). Drug concentrations of 10^{-4}M caused a reduction in phase 4 depolarization and stopped normal automatic firing.

In order to more completely understand the effects of the MDIs on the slow calcium current, transmembrane potentials were recorded from potassium-depolarized ($[K^+]_o$ = 22mM) canine Purkinje fibers after equilibration with norepinephrine (10^{-7}M) and tetrodotoxin (1 mg/L) or lidocaine (10^{-5}M), or alternatively by superfusing with sodium-free solution in which tetraethylammonium is used to replace all sodium ions. Membrane depolarization, tetrodotoxin, and lidocaine depress the sodium channels, while catecholamines augment transmembrane calcium flux (slow response) and restore regenerative propagated action potentials.[95-97] Similarly, propagated calcium-dependent slow response action potentials can be obtained in canine cardiac Purkinje fibers exposed to sodium-free solutions containing no inorganic cation other than calcium or potassium.[98] Previous studies have demonstrated the selective inhibitory properties of cobalt, lanthanum, manganese, verapamil, D600, nifedipine, and diltiazem upon electrical activity generated using the two methods described above. Based upon this apparent ability to inhibit calcium-dependent currents, these compounds are categorized as slow channel blockers.[99-101] At concentrations ranging from 10^{-5} to $5x10^{-4}$M, pr-MDI and bu-MDI reduced or abolished slow response activity in driven and nondriven Purkinje fibers superfused with K^+ (22 mM), tetrodotoxin, and norepinephrine, or in sodium-free high calcium (16 mM) solutions.

On the basis of these electrophysiological observations, it is hypothesized that the MDIs interfere with transmembrane electrical activity in canine cardiac tissue. This interference does not appear to be ionically selective in that evidence exists for interference with both sodium and calcium currents.

Because we originally assigned an intracellular site of action to the MDIs based on pharmacological[74,75,82,89] and biochemical[82] studies, the quaternary ammonium derivative of bu-MDI (2-n-butyl-3-trimethylammonium-MDI; Q-bu-MDI) was developed with the expectation of producing an inactive compound. However, electrophysiological and pharmacological studies (see below) demonstrated that Q-bu-MDI was qualitatively similar to, and quantitatively more potent than, pr-MDI and bu-MDI in most situations.

Applied Pharmacological Studies with the MDIs - Calcium antagonists are known to decrease contractility of vascular smooth muscle and the myocardium.[102] The value of this class of pharmacological agents in coronary therapeutics has been attributed to their ability to reduce myocardial oxygen consumption, decrease arterial blood pressure, and improve myocardial oxygen supply through dilation of extramural coronary arteries, collaterals, and anastamoses.[19] The pr- and bu-MDIs ($5x10^{-6}$ to 10^{-4}M) produced a prolonged, concentration-dependent relaxation of potassium-depolarized strips of bovine extramural coronary vessels, which was

reversible upon elevation of the calcium concentration of the medium.[76] The effect was qualitatively similar to that observed with prenylamine[76] and other members of the prenylamine group[19] and dissimilar to the short-lived action of the nitrites.[19] In the nonstimulated isolated perfused rabbit heart preparation, pr-MDI ($3x10^{-5}$M) and bu-MDI (3×10^{-5} to 10^{-4}M) increased coronary flow, decreased inotropic activity, and did not alter chronotropic properties of the heart.[76] Similar results are obtainable with the prenylamine group of drugs,[19,76,103] many members of which enjoy extensive therapeutic use outside the USA in the management of angina pectoris of effort,[103] as well as variant Prinzmetal angina.[104]

The antiarrhythmic properties of the MDIs were investigated in several animal models. Ouabain-induced ventricular arrhythmias in dogs could be reversed by i.v. doses of 5-30 mg/kg of either pr-MDI or bu-MDI; the reversion being preceded by a drop in diastolic blood pressure, with no appreciable alteration in systolic pressure or heart rate.[77] Pretreatment of dogs with pr- or bu-MDI also afforded significant protection against ouabain-induced arrhythmias[77] and calcium-induced arrhythmias.[78] Pretreatment of rats with 3.75 mg/kg i.v. of either pr-MDI or bu-MDI provided virtually complete protection against calcium-induced bradycardia, arrhythmias, and death.[78] The antiarrhythmic potency of the MDIs in rats was equivalent to that of verapamil, but the latter drug produced bradycardia, EKG alterations, and AV-block, whereas the MDIs exhibited no intrinsic deleterious effects on cardiac function.[78] Prenylamine and phenytoin exhibited no protective action against calcium-induced arrhythmias in rats.[78] Pretreatment of rats with the MDIs afforded significant protection against aconitine-induced arrhythmias, the order of potency being Q-bu-MDI (0.25 mg/kg, i.v.), quinidine (8 mg/kg, i.v.), bu-MDI (16 mg/kg, i.v.), and pr-MDI (24 mg/kg, i.v.).[105] Pretreatment of rats with the MDIs also afforded significant protection against methacholine-induced arrhythmias, the order of potency being Q-bu-MDI (1 mg/kg, i.v.), bu-MDI (2 mg/kg, i.v.), quinidine (4 mg/kg, i.v.), and pr-MDI (8 mg/kg, i.v.).[105] In the chloroform-anoxia assay, pretreatment of mice with the MDIs afforded at least a 70% protection against arrhythmias, the antiarrhythmic ED50s being: Q-bu-MDI (10.5 mg/kg, i.p.), bu-MDI (44 mg/kg, i.p.), quinidine (67 mg/kg, i.p.), and pr-MDI (68 mg/kg, i.p.).[105] The significant contribution of the calcium antagonists to the therapy of cardiac arrhythmias has recently been reviewed.[1,106,107]

In the heart, the action of histamine on H_1-receptors causes an increase in conduction time through the AV-node and an increase in coronary blood flow, whereas its action on H_2-receptors augments the force of contraction, increases cardiac rate and automaticity, and potentiates digitalis toxicity.[108-112] H_1- and H_2-receptor antagonists have been shown to protect the heart against ouabain-induced arrhythmia[108,109] and to block the cardiac effects of histamine and other H_1- and H_2-agonists.[110,111] Recently it has been demonstrated[113] that histamine-activated cardiac adenylate cyclase (which has the properties of an H_2-receptor) is blocked by the specific H_2-antagonist cimetidine ($pA_2 = 6.1$), as well as by the calcium antagonists L-cis-diltiazem ($pA_2 = 6.94$), verapamil ($pA_2 = 5.44$), perhexiline ($pA_2 = 5.58$), and pr-MDI ($pA_2 = 6.46$). The contribution of this H_2-blocking property of the calcium antagonists to their antiarrhythmic actions is not currently known since it neither corresponded to their calcium antagonistic potencies nor to their ability to block the positive inotropic action of histamine.[113]

The effect of calcium antagonists on platelet function is very complex. Platelet activation by various stimuli appears to be initiated by the stimulus-induced translocation of intracellular calcium stores, which sets in motion an integrated set of responses involving energy-producing reactions, contraction of actomyosin-containing filaments, alteration of microtubule assembly and distribution, release of granules containing stored products which facilitate aggregation and clotting, change in membrane properties leading to cellular adhesion, activation of the synthesis of PGs, PG endoperoxides, and thromboxane A_2, and the appearance of procoagulant platelet factor 3 in the surface membrane.[114] The calcium antagonist TMB-8 has been shown to reduce platelet secretion and aggregation induced by thrombin, ionophores, and ADP.[115,116] On the other hand, at concentrations up to 0.5 mM, pr-MDI induced platelet secretion and thus may act as a calcium agonist in this system.[115] This latter action is reminiscent of the action of verapamil on skeletal muscle where the drug itself induces contracture and also potentiates caffeine-induced contractures.[117]

Toxicological Studies with the MDIs - The acute and subacute toxicological profiles of the MDIs were recently reported from our laboratories.[83] Acute toxicity studies resulted, in mice, in an i.v. LD50 of 40 and 32 mg/kg for pr-MDI and bu-MDI, respectively, and an i.p. LD50 of 185 mg/kg for both MDIs. In rats, the i.p. LD50 was 175 and 240 mg/kg for pr-MDI and bu-MDI, respectively. In view of the antiarrhythmic equipotency of verapamil, and the MDIs in the calcium-induced arrhythmia model in rats,[78] it is clear that the therapeutic ratio of the MDIs is superior to that of verapamil since the latter exhibits an LD50 of 67 mg/kg (i.p.) and 16 mg/kg (i.v.) in rats and 68 mg/kg (i.p.) and 6.7 mg/kg (i.v.) in mice.[118] Likewise, the therapeutic indices of the MDIs in the chloroform-anoxia assay in mice compare favorably with that of quinidine,[105] the latter having an LD50 of 225 mg/kg (i.p.).[119] The LD50 of Q-bu-MDI in mice is 65 mg/kg (i.p.).

An i.v. dose of 16 mg/kg of pr-MDI or bu-MDI decreased motor activity and prolonged barbiturate sleeping time in mice, but did not affect conditioned avoidance behavior or motor coordination tests.[83] In subacute toxicity studies,[83] rats received daily for 4 weeks 26.25 or 52.5 mg/kg i.p. of each of the pr-MDI or bu-MDI, while mice received 23.13 or 46.25 mg/kg i.p. of either MDI. No alterations were observed in serum alkaline phosphatase, glutamic-pyruvic transaminase, glutamic-oxalacetic transaminase, creatine phosphokinase, bilirubin, chloride, cholesterol, uric acid, prothrombin time, and bromsulphalein retention. Blood glucose was slightly lowered, and so was serum calcium in male mice. The higher dose of pr-MDI elevated serum lactate dehydrogenase in rats. Both MDIs elevated serum isocitric dehydrogenase in male rats. However, light microscopic examination of brain, kidney, liver, spleen, intestine, stomach, and myocardium showed no anomalies resulting from the 4-week MDI treatment, and electron microscopic examination of hepatocytes revealed no deleterious effects of either MDI.[83]

Structure-Activity Studies on the MDIs - Structure-activity studies done so far in our laboratories can be summarized as follows: Shortening the 2-substituent (to produce 2-methyl- and 2-ethyl-3-dimethylamino-MDIs) resulted in reduction of calcium antagonistic activity.[74] Lengthening the 2-substituent or rendering it more bulky (2-n-heptyl-, 2-cyclohexyl-, and 2-phenyl-3-dimethylamino-MDIs) resulted in reduction or loss of calcium-antagonistic activity and emergence of agonist activity.[81] The agonist activity of the phenyl analogue on smooth muscle was not blocked by

atropine (a muscarinic receptor blocker) or propyl-dibenzyloxyindanpropionic acid (a $PGF_{2\alpha}$ receptor blocker[80]), but was blocked by bu-MDI and by prenylamine, indicating an involvement of increased calcium influx.[81] Opening the 5,6-methylenedioxy bridge (with resultant formation of the corresponding 5,6-dimethoxy analogues) results in reduction of calcium antagonistic properties and emergence of agonist activity.[79] Conversion of bu-MDI to the quaternary ammonium derivative (Q-bu-MDI) resulted in significantly enhanced potency.[105] Recent studies have demonstrated that the quaternary ammonium compounds, pranolium[120] and bretylium,[121] localize in cardiac muscle and that their myocardial tissue concentrations, not plasma concentrations, correlate with their antiarrhythmic effects. Finally, conversion of pr-MDI to propyl-dibenzyloxyindanpropionic acid resulted in the emergence of selective $PGF_{2\alpha}$ blocking activity at low concentrations and retention of calcium antagonistic activity at high concentrations (in the mouse ileum).[80]

Conclusions - No single pharmacological group of agents has generated as much recent interest as the calcium antagonists. As experimental tools, the calcium antagonists have contributed enormously to expanding the frontiers of pharmacology and physiology. As therapeutic agents they hold considerable promise particularly for the management of cardiovascular pathologies. However, despite a significant investment in basic research aimed at elucidating the complex nature of their cellular effects, their pharmacological mechanism of action remains to be unraveled.

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Chapter 24. Recent Advances in Parasite Biochemistry

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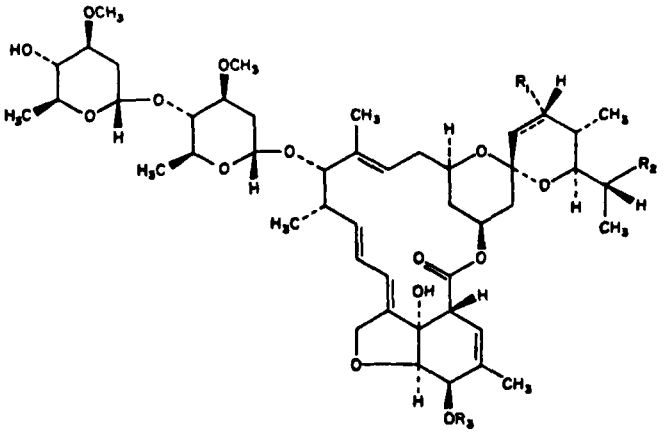
In the past year, there has been significant progress made in the biochemical investigations of various parasites, which are the major causes of tropical diseases in humans as well as animals. Most of these studies take a comparative approach to specify unique biochemical components, activities and metabolic pathways in the parasites, or to understand the biochemical mechanisms of action among some of the known antiparasitic agents. The results of these research activities will no doubt bring closer the possible realization of rational pursuit of prophylactic and chemotherapeutic controls of parasitic diseases. A few review articles have appeared recently on the subject of biochemistry of Plasmodium,[1] new biochemical strategies in chemotherapy of parasitic worms[2,3] and kinetoplast DNA in trypanosomatids.[4] In the present review we shall cover subjects on the modes of action of avermectins and praziquantel, purine and pyrimidine metabolism in protozoan parasites, polyamines in hemoflagellates, carbohydrate metabolism and surface glycoprotein in African trypanosomes, and kinetoplast DNA in kinetoplastida.

Avermectins - A family of structurally related macrocylic lactone disaccharides (1), isolated from the mycelia of Streptomyces avermitilis,[5] has demonstrated highly potent and broad spectrum anthelminitic activities.[6] The component avermectin B_{1a} (AVM) is highly efficacious against parasitic nematodes Haemonchus contortus, Ostertagia ostertagi, Trichostrongylus colubriformis, Trichostrongylus axei, Cooperia oncophora, Cooperia punctata, Trichostrongylus longispicularis, Dictyocaulus viviparus and Oseophagostomum radiatum in cattle; near total control of these parasites was attained at dosage levels of 50 to 200 μg/kg of body weight by various means of administration.[7,8] It also caused rapid removal of Diofilaria immitis microfilariae from the blood of infected dogs at similar doses.[9] A chemical derivative 22,23-dihydroavermectin B_1, otherwise known as ivermectin, was prepared from avermectin B_1 by selective hydrogenation using Wilkinson's homogeneous catalyst [$(Ph_3P)_3$ RhCl],[10] and possesses anthelmintic activities,[11-14] and activities against the ticks Rhipicephalus sanguineus and Dermacentor albipictus,[13] similar to the parent compounds. The avermectins have been found also active against various insects; they controlled Tribolium confusum totally at 100 ppm



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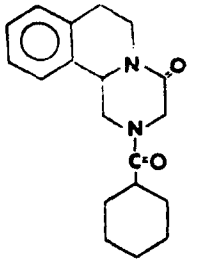
in the feeding medium, completely prevented larval breathing holes of Cuterebra spp. in mice at 0.1 mg per kg body weight[15] and have LC_{50} values of 0.0058 to 0.03 ppm against the sheep blowfly Lucilia cuprina.[16]

The avermectins act on nematodes by rapid immobilization of the worms without affecting their normal muscular tone.[17] AVM has no effect on the contractility of dorsal muscle strip of Ascaris,[18] and exerts equal paralyzing effects on the wild type and levamisole-resistant mutant of the free-living earth worm Caenorhabditis elegans,[17] which suggest that AVM does not act on the cholinergic neuromuscular junctions of the two nematodes on which levamisole acts as an acetylcholine agonist. Further studies of AVM effects at the neuromuscular junction of the lobster walking leg indicated that the drug inhibited both excitatory and inhibitory postsynaptic potentials by decreasing the muscular membrane resistance.[18] The change was caused by opening of the chloride ion channels on the muscle membrane, and could be reversed by the γ-aminobutyric acid (GABA) antagonist - chloride ion channel blocker picrotoxin.[18] A similar observation was made in the Ascaris nervous system; AVM blocked the signal transmission from the ventral interneurons to the excitatory motor neurons, and this block could be reversed by picrotoxin.[17] AVM also decreased the inhibitory motor neuron signals at the Ascaris neuromuscular junction which presumably also has GABA as the transmitter.[17] It is thus tentatively concluded that the avermectins may potentiate GABA actions in arthropods and nematodes and such potentiation may be the mechanism of the insecticidal and anthelmintic actions.

Upon further investigation, AVM was found to have a specific stimulatory effect on GABA release from preloaded rat brain synaptosomes.[19] It also potentiates specific GABA binding to the washed rat brain membranes by increasing the number of GABA binding sites;[20] the increase is dependent on the presence of chloride ion and is blocked by picrotoxin. Both the AVM activities explain adequately the neurophysiologicial effects of the drug. AVM is also known to bind specifically to brain synaptosomes with high affinity, and the specific binding is concentrated mainly in the cerebellum where the GABA nerve terminals have a relatively high density.[21] Like GABA, AVM potentiates benzodiazepine binding to brain membranes,[22,23] and enhances some of the pharmacological actions of diazepam in vivo. It has become apparent that AVM could be used as a powerful tool in studying GABA nerves.

Praziquantel - A novel antischistosomal agent[24] and cestocide,[25] which has a chemical structure (2) resembling a benzodiazepine, causes spastic paralysis of parasite musculature.[25] The benzodiazepine derivatives clonazepam (3)[26] and Roll-3128 (4),[27] both having central nervous effects similar to those of other benzodiazepines, are potent



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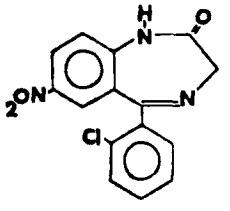

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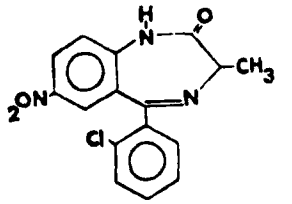

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antischistosomal drugs, and act similarly by paralyzing the parasites via muscular contraction. The activities of these three compounds are particularly interesting since there has been no indication in schistosomes of GABA nerves on which the benzodiazepines are known to act.[28] Furthermore, these benzodiazepines, acting as muscle relaxants in mammals, cause an opposite effect of muscular contraction in the parasites. Various compounds known to interact with schistosomes neuroreceptive sites did not affect the actions of these benzodiazepines.[27] Depolarization of the tegument membrane of adult male Schistosoma mansoni was induced by praziquantel, but it was too limited and too slow to be the cause of spastic paralysis.[29] Praziquantel and Roll-3128 decrease the influx of K^+ but stimulate the influx of Ca^{++} and Na^+ into the male schistosome.[27] Removal of Ca^{++} or addition of Mg^{++} to the incubating medium blocked the action of these drugs on the parasite musculature,[27] but the Ca^{++} antagonist D-600, Co^{++} and La^{+++} exert no significant inhibition on either Ca^{++} influx or muscular contraction in schistosomes induced by praziquantel.[30] Further observation that ouabain, a specific inhibitor of Na^+K^+-ATPase, also caused a pronounced tension increase of schistosomal musculature has led to studies of competition between praziquantel or Roll-3128 and ouabain for binding to the receptors on the surface of male S. mansoni.[31] The results revealed high affinity binding of the two antischistosomal agents to ouabain receptors,[31] which are most likely the Na^+K^+ pump of the parasite. It is thus possible that the mechanism of antischistosomal action of praziquantel and its analogs is by inhibiting the Na^+K^+ pump of schistosomes. This contention is supported by the finding that close structural analogs, such as Bay-4973 and diazepam, which lack antischistosomal activity, also lack the ability to interact with the ouabain receptor.[31]

Recent microscopic studies have indicated that praziquantel primarily vacuolizes the tegument of S. mansoni,[32] Dicrocoelium dendriticum[32] and the dwarf tapeworm Hymenolepis nana.[33] The vacuolization of tegument occurs within a short time (5 min.) at a low dose of praziquantel (1 µg/ml) in vitro, and eventually leads to the disruption of the apical tegumental layer. It is not known whether these changes are related to the other praziquantel effects mentioned in the previous discussion.

Purine and Pyrimidine Metabolism in Protozoan Parasites - There has been mounting interest in the purine and pyrimidine metabolism of protozoan parasites during recent years because of the many unique aspects of these pathways which are just being gradually recognized.

(1) Dihydrofolate reductase and thymidylate synthetase: These two enzymes, identified as two separate proteins from most organisms and mammalian sources with molecular weights of 18,000-22,000 and 60,000-70,000 (dimer), respectively, have been isolated from various protozoan parasites both with high molecular weights of 100,000 to 240,000 by gel filtration.[34] There have been also indications that levels of the two enzymes in Plasmodium chabaudi varied in parallel during the course of a synchronous infection.[35] The two enzyme activities of Plasmodium berghei were co-purified on Sephadex G-200 and methotrexate Sepharose columns.[34] Dihydrofolate reductase from the protozoal flagellate Crithidia fasciculata has been recently purified to homogeneity, and found to be a bifunctional protein with thymidylate synthetase activity as well.[34] The native protein has a molecular weight of 107,000 and a monomer molecular weight of 56,700 ± 200.[34] These findings suggest the possibility that most, if not all, of the protozoan parasites may

have dihydrofate reductase and thymidylate synthetase in a single polypeptide with independent sites for catalysis for the two reactions. Presumably it will result in advantages to the cells in the regulation or efficiency of these reactions.[36] It may also provide an attractive target for chemotherapeutic control of the parasites, which may have been verified by the past discoveries of many antifolates and pyrimidine analogs as effective antiprotozoan agents.[37]

(2) Pyrimidine metabolism: All parasitic protozoa examined so far have demonstrated capabilities of incorporating bicarbonate and orotate into nucleic acid pyrimidine rings,[38] which suggests that they can perform at least part of the pyrimidine de novo synthesis. The conversion of dihydroorotate to orotate has been recently studied in Kinetoplastida[39,40] and Plasmodium.[39] The responsible enzyme in Kinetoplastida is a hydroxylase located in the cytoplasm of the cell and probably requires tetrahydrobiopterin for activity, whereas that in Plasmodium is most likely a dehydrogenase in mitochondria positioned at the ubiquinone level, and can be inhibited by menoctone. The orotate phosphoribosyl transferase and ortidylate decarboxylase activities were identified in the particulate fractions of Leishmania mexicana,[40] C. fasciculata[40] and the crude extracts of Plasmodium berghei.[41] The promastigote of L. mexicana has a 40 times higher level of the two enzyme activities than that in the amastigote,[40] which may suggest more vigorous de novo pyrimidine synthesis in the parasite when it is in the sand fly vector. The P. berghei orotate phosphoribosyl transferase activity is apparently rate-limiting, and can be inhibited by 5-azaortate > 5-azauracil > 6-azauracil > oxipurinol > allopurinol.[41] These findings indicate potential value of the enzymes for antiprotozoan chemotherapy.

Many protozoan parasites, such as Plasmodium, are unable to salvage pyrimidines and are thus totally dependent on de novo synthesis.[38] However, Toxoplasma gondii is known to possess uracil phosphoribosyl transferase, uridine and deoxyuridine phosphorylase and to be able to salvage uracil.[42,43] This ability is lost in one 5-fluorodeoxyuridine-resistant mutant which has a hundred-fold lower concentration of uracil phosphoribosyl transferase.[42] T. gondii thus can probably survive solely on de novo pyrimidine synthesis. Uracil and orotate phosphoribosyl transferases must be either two different enzymes in T. gondii, or the mutation must have altered the substrate specificity of a single enzyme to recognize both uracil and orotate to recognizing orotate only. In any case, simultaneous inhibition of both uracil and orotate phosphoribosyl transferase activities should elicit effective anticoccidial chemotherapy. A recent finding that some 6-azauracil derivatives, each having an aryl group attached to the N^1-position, have more potent anticoccidial activities than 6-azauracil has generated interest,[44] because these derivatives are actually weaker inhibitors of orotidylate decarboxylase which is presumably the target enzyme for 6-azauridylate formed from 6-azauracil It is possible that the coccidial orotidylate decarboxylase may not be the target of these derivatives. Uracil phosphoribosyl transferase, orotate phosphoribosyl transferase, or both could be the real therapeutic target.

(3) Purine metabolism; All the protozoan parasites examined to date appear to be incapable of synthesizing purines de novo, because of the failure of radiolabeled glycine and formate to label the purines of the parasites.[45,46,47,48] Hypoxanthine, adenine and adenosine have been regarded as the three major sources of purines for these parasites,[49] but recent evidence increasingly favors the hypothesis that hypoxanthine may

be the main, if not the only, supply of purines to Plasmodia,[1,50] Leishmania,[45,51] C. fasciculata,[51] Toxoplasma gondii[52] and Eimeria tenella.[47] Allopurinol, an analog of hypoxanthine nontoxic for mammalian cells, was found effective in vitro against Leishmania,[53] Trypanosoma cruzi[54] and African trypanosomes[55] by being coverted sequentially in the parasites to allopurinol ribonucleotide, aminopurinol ribonucleoside mono-, di- and triphosphates, and eventually incorporated into these organisms' RNA.[56] The antiparasitic activities and the metabolism of allopurinol can be reversed by hypoxanthine, inosine and adenine, suggesting that the hypoxanthine salvage pathway in the parasites may be responsible for the activities by recognizing allopurinol and its derivatives as substrates. Purine phosphoribosyl transferases from Leishmania donovani promastigotes were analyzed,[57] and found to consist of three individual enzymes, hypoxanthine-guanine, xanthine and adenine phosphoribosyl transferases. The hypoxanthine-guanine phosphoribosyl transferase has a high pI value of 7.0, accepts allopurinol as a 68-fold less efficient substrate than hypoxanthine and is much less susceptible to the 8-azapurine analogs than the human enzyme. C. fasciculata, also sensitive to allopurinol in vitro,[58] has hypoxanthine, guanine and adenine phosphoribosyl transferase activities in three separate enzymes.[59] It is not clear which of the three enzymes utilizes allopurinol as substrate. Allopurinol is also known to control Eimeria tenella growing in cell cultures.[60] Hypoxanthine, xanthine and guanine phosphoribosyl transferases from unsporulated oocysts of E. tenella have been recently purified to homogeneity by GMP-agarose column chromatography.[61] It turned out that all the three activities are in one enzyme (HXGPRTase) with a subunit molecular weight of about 23,000.[61] Like the leishmanial enzyme, E. tenella HXGPRTase has a relatively high pI and a low susceptibility to 8-azaguanine. Allopurinol competes with hypoxanthine (Km = 0.25 ± 0.11 μM) for the enzyme with a K_I value of 21.5 ± 9.4 μM. It is apparent that an effective inhibition of purine phosphoribosyl transferases should prevent growth of these parasitic protozoa. Some studies have been already initiated recently to design specific inhibitors of these enzymes for antiparasitic purposes.[62]

Adenylosuccinate synthetase and lyase were also isolated from promastigotes of L. donovani.[63] Allopurinol ribonucleotide is a substrate of the synthetase with about a 30-fold lower affinity and a 100-times slower reaction rate than those of IMP.[63] Succino-allopurinol ribonucleotide was efficiently cleaved by the parasite lyase with somewhat higher Km and lower Vmax than adenylosuccinate.[63] It also acts as a competitive inhibitor versus adenylosuccinate with a K_I value of 31.3 ± 2.2 μM.[63]

Allopurinol ribonucleoside was shown recently to be more effective than allopurinol in inhibiting the growth of Leishmania promastigotes in vitro.[64] Both compounds were equally effective in preventing the transformation of amastigotes to promastigotes of L. donovani.[64] The mechanism of action of the ribonucleoside is via its conversion to 5'-nucleotide catalyzed by a nucleoside phosphotransferase in the parasite. The enzyme can utilize TMP, p-nitrophenylphosphate, UMP and thymidine 3'5'-diphosphate as the phosphate donor but cannot use ATP. It may be possible to exploit this unusual enzymatic activity in developing a new chemotherapeutic approach for treating leishmaniasis. Allopurinol ribonucleoside is nontoxic to mammalian cells and is resistant to nucleoside cleavage both in man and Leishmania promastigotes.

A plant growth regulator, N^6-methyladenine, has been found to inhibit the growth of Trypanosoma cruzi and Leishmania in vitro at millimolar concentrations.[65] The extent of growth inhibition is related to the sensitivity of guanine deaminase to N^6-methyladenine in these organisms. It is unclear, however, how inhibition of the enzyme would lead to inhibition of growth.

An adenine derivative, arprinocid (9-(2-chloro-6-fluorobenzyl) adenine), has been found to be a potent anticoccidial agent.[66] It acts by blocking the transport of hypoxanthine into the parasites.[67,68] It has an ED_{50} value of about 20 ppm against the growth of E. tenella in cell cultures and the activity is apparently reversible by hypoxanthine.[69] Hypoxanthine transport in HeLa cells[68] and Chinese hamster lung fibroblasts[70] was also competitively inhibited by arprinocid with K_I values between 30 and 40 μM. Under in vivo conditions, arprinocid is partly converted by microsomal metabolism to arprinocid-1-N-oxide which is 60-times more potent than arprinocid in controlling E. tenella in cell cultures.[70] Its mode of action is probably by dilating the rough endoplasmic reticulum membranes in the parasite via further microsomal metabolism.[71]

Polyamines in Hemoflagellates - Putrescine and spermidine are present in the promastigotes of trypanosomatids at relatively high levels,[72] e.g., L. donovani promastigotes contained the two polyamines at 30 to 40 nmoles each per mg sample protein.[73] The levels fluctuate during the growth cycle; maximal levels being attained during the logarithmic phase.[74,75] In L. donovani amastigotes, the amount of putrescine is dropped by some 17-fold with the appearance of some spermine.[73] Ornithine decarboxylase[73,76] and S-adenosyl-L-methionine decarboxylase[77] activities are present in all forms of hemoflagellates so far examined. and fluctuate with the growing phases of the parasites.[77] There is only limited uptake of exogenous polyamines by T. brucei,[72] suggesting dependence of the parasites on endogenous synthesis of polyamines which may be essential for parasite growth.

The polyamines, particularly spermidine at 0.1 to 0.2 mM, activate NAD-α-glycerophosphate dehydrogenase from the insect trypanosomatid Leptomonas.[78] Cationic trypanocides, such as Antrycide and ethidium bromides interfere with the polyamine stimulation with K_I values in the 0.1 to 0.5 mM range.[79] There are two types of DNA polymerases from T. brucei,[80,81,82] both are immunologically distinct from the host enzymes.[80,81] One of those two T. brucei enzymes, the Peak I, is stimulated up to 4-fold by spermine and spermidine at millimolar concentrations.[82] Similar concentrations of spermine and spermidine were also found inhibitory of a certain protein kinase I from T. brucei.[83] These biochemical effects of polyamines on the hemoflagellates may be interesting, but since they all take place at equally high concentrations of the polyamines, it is difficult to distinguish which function(s) of the polyamines is essential for the parasites. In T. brucei-infected mice under treatment of the cationic trypanocidal agents amicarbalide, imidocarb, Antrycide, isometamidium, pentamidine or prothidium, further administration of spermidine at 300 mg per kg body weight or spermine at 100 mg per kg has effectively negated the antitrypanosomal activities of all the drugs.[84] It seems unlikely that all of these drugs would act on T. brucei by interfering with the essential function(s) of spermidine and spermine, especially since T. brucei uptake of the polyamines is very limited.[72] It appears more likely that the cationic polyamines may block the transport of the cationic trypanocides into T. brucei. The reverse

may be also true in view of the moderate inhibition of putrescine uptake in Leishmania spp. promastigotes by ethidium bromide and pentamidine.[85]

A potent inhibitor of mammalian and yeast S-adenosylmethionine decarboxylase, methylglyoxal-bis(guanylhydrazone) (MGBG),[86] is known to possess anti-hemoflagellate activity[87] and inhibitory effects on hemoflagellate S-adenosylmethionine decarboxylase.[77] At the concentration of 1.2 μM, MGBG inhibits about 50% of the growth of L. donovani promastigote after 20 hr. incubation; the same experiment also revealed significant decreases of spermine and spermidine levels in the parasite.[85] These results support the contention that MGBG controls hemoflagellates by inhibiting their polyamine synthesis. α-Difluoro-methylornithine, a specific and irreversible inhibitor of ornithine decarboxylase, was found recently to decrease incorporation of radiolabeled L-ornithine into putrescine in T. brucei with an ID_{50} value around 0.05 mM.[88] When T. brucei-infected mice were dosed with 1% of the compound in drinking water for 3 days, the parasites were brought under total control.[88] It is thus clear that blockage of polyamine synthesis appears to be an attractive rational approach to chemotherapy of hemoflagellates.

Glycolysis in Trypanosoma brucei - In the bloodstream forms of T brucei, a number of glycolytic enzymes, hexokinase, phosphoglucoseisomerase, 6-phosphofructokinase, fructose-1,6-diphosphate aldolase, triosphosphate isomerase, glycerol kinase and phosphoglycerate kinase are all confined in a microbody-like organelle; the glycosome.[89] The multienzyme complex sediments at 14,500 x g, emerges in the void volume of Bio-Gel A-5m column, and bands at a density of 1.22[90] to 1.23 gm/ml.[89] It is surrounded by membrane structure,[89] and exhibits latency of enzyme activities; up to 90% of which can be abolished by Triton X-100,[91] relatively high concentrations of digitonin[92] or by freezing and thawing.[91,92] The data suggest that the glycosomal membrane may consititute a permeability barrier to phosphorylated intermediates, and explain why T. brucei has an extremely high rate of glycolysis. But they also require that substrates and end-products of glycosomal metabolism cross the glycosomal membrane at high rates in order to make the fast glycolysis possible, since phosphoglyceromatase, enolase and pyruvate kinase are located in the cytosol,[92] and glyceraldehyde phosphate dehydrogenase and sn-glycerol-3-phosphate dehydrogenase are found in both compartments.[89] One thus would expect that dihydroxyacetone phosphate glycerol -3-phosphate and glycerate-3-phosphate may be capable of crossing glycosomal membranes. Recent studies of the partially purified T, brucei pyruvate kinase indicate fructose-1-6-diphosphate to be a heterotropic allosteric activator of the enzyme,[93] suggesting a capability of this phosphorylated intermediate to cross the glycosomal membane also. These possibilities receive some credibility from the almost 10-fold higher levels of glycolytic intermediates in T. bruei cells when compared to those in muscle, liver and erythrocytes,[92] and the similar mass-action ratios for individual glycolytic steps between T. brucei and other cell-types which have no compartmentalization of glycolysis.[92] The glycosomes, which have been also identified in T. cruzi,[94] thus make a potentially interesting target for chemotherapeutic research.

T. brucei glycolysis under anaerobic conditions generates equimolar glycerol and pyruvate from one glucose molecule with no obvious means for ATP net gain. One hypothesis calls for the possible reversal of the glycerol kinase catalyzed reaction to produce glycerol and ATP from glycerol-3-phosphate and ADP.[89] This theory is supported by a 4-fold increase of glycerol-3-phosphate content in T. brucei under anaerobic conditions,[92,95] independent of the concentration of added glycerol.[95]

Glycerol-3-phosphate accumulation also partially reverses the glycerol inhibition of T. brucei anaerobic glycolysis.[95] Further analysis of T. brucei glycosomal glycerol kinase has shown the capability of the enzyme to catalyze sn-glycerol-3-phosphate-dependent ADP phosphorylation.[96] The rate of this reaction is sufficient to account for the observed glycerol production and ATP synthesis by intact cells under anaerobic conditions.[96] Other trypanosomatids have much lower glycerol kinase activities and may not depend on this enzyme for ATP synthesis under anaerobic conditions.[96]

Surface Glycoprotein of Trypanosoma Brucei - Messenger RNA's (m-RNA's) have been extracted from bloodstream forms of cloned T. brucei S427 variants MIAG099,[97] 11E1,[98] MIAG121,[99,100] and MIAG201,[99,100] They were translated in reticulocyte lysate[97,99,100] or wheat germ extract,[98] and, in every case, a predominant polypeptide of about 60,000 molecular weight was formed. The polypeptide was specifically precipitated by antiserum raised against the purified, cloned variant antigen, and comigrated with the ^{125}I-labelled, purified variant specific antigen in electrophoresis. Polysomes carrying the variant specific surface antigen was isolated by immunoprecipitation and account for about 3% of the total polysomes.[100] The m-RNA that directed the synthesis of the surface antigen was about 2.0 kilobases long.[98] Complementary DNA (c-DNA) synthesized from the m-RNA of clone 11E1 surface antigen was used as a probe for homologous hybridization experiments. The results indicated 42% of low complexity fraction in the partially purified m-RNA. In cross-hybridization experiments to poly(A)$^+$-RNA from an unrelated clone 3E9, the proportion of low complexity sequences was much reduced but the c-DNA probe was driven to the same plateau of 93% hybridization. The result indicates that although the abundance of some of the RNA sequences is clone-specific, these sequences nevertheless exist in both clones, i.e., some of the genes coding for low complexity RNA sequences in a clone are not clone-specific, but their mechanism of expression may be clone-specific.[98] A similar study of the m-RNA coding for variant specific surface antigens from Trypanosoma brucei gambiense was also carried out recently with similar results.[101] For the two m-RNA species TTrT-1 and TTrT-17, the molecular lengths were calculated to be 1.83 and 2.1 kilobases respectively.

In another recent investigation,[102] poly(A)$^+$-RNA from four antigenic variants of one clone of T. brucei was translated in a rabbit reticulocyte lysate and gave rise to proteins that could be precipitated with antisera against homologous variant surface glycoprotein. The electrophoretic mobilities of two of the four proteins in SDS-gel appeared to be slower than would be anticipated from the un-glycosylated, mature product and thus suggested the presence of presequences as possible "signal peptide" involved in the transfer of nascent protein to cellular surface. This possibility was recently verified on one of the variant surface glycoproteins #117;[103] c-DNA molecules corresponding to the m-RNA for this antigen were cloned into the plasmid PBR322,[102] and one of the c-DNA inserts T-cV117.8 was sequenced. The nucleotide sequence suggests that the primary translation product has a hydrophobic tail of 23 amino acid residues at the carboxy terminus which is not found on the isolated, mature glycoprotein. The data also suggest that the glycosylated residue at the carboxyl terminus is aspartic acid rather than the anticipated asparagine.[103]

Poly(A)$^+$-RNA from each of the four variants[102] only hybridized to the homologous c-DNA in filter hybridizations. Colony hybridization of c-DNA to the cloned Escherichia coli showed that only 7-10% of the colonies showed hybridizaton and they hybridized only with the homologous probe. Among ten plasmid DNA preparations from ten such colonies, eight hybridized specifically with the complementary m-RNA.[102] This lack of cross-hybridization indicates that antigenic diversity in African trypanosomes, unlike antibody diversity in mammals, does not involve linkage of a repertoire of genes for the variable N-terminal half to a single gene for the C-terminal half of the surface glycoprotein.[102]

Kinetoplast DNA in Kinetoplastida - There have been large numbers of analyses by restriction endonuclease digestion on the physical maps of maxicircles and minicircles in kinetoplast DNA of Leishmania tarentolae,[104,105] Trypanosoma equiperdum,[106,107,108] Trypanosoma brucei[109,110] and Trypanosoma cruzi[111] in recent years. These studies point out the complexity of the DNA structures and the heterogeneity of the minicircles without clarifying probable biological functions of these circular DNA's. One study was able to group T. cruzi in subpopulations according to their minicircle DNA restriction maps as a simple method for characterizing different strains.[112]

In Crithidia luciliae, cellular RNA was found to hybridize with only the restricted maxicircle DNA, predominantly with a segment of only 2300-2500 base pairs.[113] Two kinetoplast RNA species of 9S and 12S were isolated from L. tarentolae and found to hybridize selectively to the maxicircle kinetoplast DNA.[114] Two similarly small kinetoplast RNA's were identified in T. brucei recently; they also hybridize to the maxicircle component of kinetoplast DNA, specifically to the smaller cloned Hind III maxicircle fragment.[115] The fragment had substantial sequence homology with that from L. tarentolae containing the 9 and 12S genes.[115] It is thus likely that the maxicircle DNA is at least partially transcribed and that there may be an evolutionary conservation of the 9 and 12S gene sequences.

The total sequences of two kinetoplast DNA minicircles of T. brucei have been determined recently.[116] They share about 27% of their sequences with the largest continuous region of 122 base pairs. Both sequences contain a large number of translation termination codons in all potential translation reading frames. The largest oligopeptide potentially specified by one minicircle sequence is 52 amino acids; the largest by the other minicircle sequence is 71 amino acids. It was proposed that massive recombination among the interlocked minicircles in the kinetoplast DNA network may account for the homology in the two minicircle sequences,[116] but the real function of these minicircles remains unknown.

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Section VI - Topics in Chemistry and Drug Design

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Chapter 25. Computer-Directed Organic Synthetic Analysis

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Introduction - An earlier chapter in this series[1] has dealt with computer-assisted organic synthetic analysis. The term "organic synthetic analysis" describes the process by which one or more potential synthesis routes are selected for a given organic compound. The term "computer-assisted" is an implication that this selection process is a task to be performed by a chemist, in which the computer can be of some assistance in its capacity as a manipulator of chemical information.[2] If it is possible for us humans to eschew for a moment our anthropocentrism, we must admit to the possibility that all functions of the human intellect can be described as manipulations of information. If such be the case, then organic synthetic "analysis" is as likely a task for a computer as it is for a human brain. The discussion that follows will focus then on those aspects of current research that deal with computer-directed organic synthetic analysis or computer-directed synthesis route discovery - since the synthesis routes already exist, in a sense, and the computer is merely discovering them.

Exploration of the possibilities of simulating complex human intellectual processes with computer programs is generally classified by computer scientists as artificial intelligence research.[3] Stepwise intellectual processes can be abstractly represented as a type of graph called a tree. The branch points in this tree are equivalent to points in the process where decisions are required. Finding a good synthesis pathway for a particular organic molecule can be looked at as an exploration of the tree composed of all possible chemical synthesis pathways leading to that molecule. Given the number of known chemical transformations, the tree of pathways leading to a moderately complex organic molecule grows at such a rate at each step that examining pathways of more than three or four steps is not computationally reasonable with even the fastest available computers.[4] We recognize, however, that chemists do not deal with this problem in such a prohibitively "expensive" way. Most often, a chemist will mentally explore a few likely lines of synthesis without even pretending to having considered all the alternatives at each point. It is this notion of an "intuitive" shortcut that has inspired much of the work in the subdiscipline of artificial intelligence research known as heuristic programming. Clearly, heuristic programming offers a way to overcome this obstacle.

What sorts of criteria can be used to select between two organic synthesis pathways? In general, the highest yield from the cheapest starting materials is most desirable. In selecting pathways, we look for high yield reactions and seek to minimize the number of steps. Convergent synthesis pathways are also preferred. Other constraints on the chemistry employed are possible, such as requiring or prohibiting the use of certain

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reagents, conditions or intermediates. Applying such criteria as these in selecting between several partially explored synthesis pathways can result in somewhat greater efficiency but neglects the equally important consideration of synthetic complexity[5,6] or synthetic direction. If, for example, two partial routes to a particular compound are being considered, and the first route, though shorter and with higher overall yield, leads to a precursor that will prove to be as difficult to synthesize as the original compound, while the second route leads to one that is easily made from available materials in only one more step, the failure to appreciate this near-solution may result in much time wasted exploring the unproductive first route. Most computer-directed synthesis discovery programs today still rely on single "cost" evaluation for heuristic guidance.

Existing Programs - There are a number of programs in existence that are designed either to solve or to assist in the solution of organic synthesis problems. Before discussing their differences, a few words should be said about their common elements.

The existing organic synthesis programs, while designed quite differently, have been built using many very similar components. Since all these programs deal with chemical molecules, all share the need to store and manipulate representations of these entities. One component common to all organic synthesis programs is an input/output facility, a communication link with the outside world. The representations of molecules used by the computer are often not very convenient for the chemist to read and vice versa; therefore, a translation facility of some type is generally indicated. The exact form of such a facility varies according to the kinds of peripheral input/output hardware devices available at a particular computer installation. Output is most often in the form of drawings or pictures of molecules along with some abstract expression of their relationships to one another on the proposed synthesis pathways and some textual information describing the nature of the chemical transformations involved. Input can vary from a simple linear shorthand description of the target molecule to executing a drawing of the target molecule using an interactive graphics terminal.

The basic description of a molecule, from a computational point of view, requires that the component atoms and their connections to one another be specified. For convenience in storing and retrieving these representations of molecular information, it is very useful to derive a unique linear descriptor for each - equivalent to the IUPAC names for compounds used for indexing the chemical literature. The most important manipulation that these programs perform on the stored molecular descriptions is chemical transformation. A copy of a particular molecule's representation can be transformed into a representation of a possible precursor by following a stored set of rules describing this procedure. The collection of transformation rules available to a particular synthesis program ideally spans all chemical knowledge and is variously referred to as a reaction library, chemical transform file, etc. Other information about the molecule that can be calculated, at least in principle, from this basic topological description, includes 3-dimensional coordinates of all atoms, bond angles and lengths, electron densities and non-bonded distances. Perception of chemically defined features, such as functional groups and rings, is also of vital importance, as most of the useful chemical information we have is expressed in terms of such constructs.

It may be well to comment briefly on the distinction that is often made between interactive and batch programs. Those with only a slight

familiarity with computer terminology often interpret these terms to be mutually exclusive and decide that the former, which seems inherently more flexible, is always to be preferred. In fact, any "batch" program that is capable of performing an independent analysis of possible synthesis pathways can easily be modified to allow interruptions in its execution during which a chemist may influence its evaluation process. The degree to which this stop/modify/restart process is perceived by the chemist to be "interactive" is largely dependent on the nature of the hardware and the operating system software. Those programs that were originally designed for exclusively interactive use have been or can be modified to run without guidance, but such modification may require a substantial effort.

Perhaps the most aesthetically appealing approach to organic synthesis route discovery is the one first described in Ugi[7] in 1971, as embodied by the CICLOPS program and in more recently developed programs such as EROS[8] and AHMOS.[9] This approach is based on a description of organic chemistry that groups all known synthetic organic chemical reactions into one of thirty-eight mechanistic categories.[10,11] This number has recently been extended to forty-three by Bart and Garagnani.[12] The notion of spanning all chemical reactions with a small set of generalized transformations is a neat and economical way of constructing a reaction library. The advantage of being able to predict every possible precursor for a given molecule must be weighed, however, against the truly staggering numbers of such precursors that can be produced for molecules of even modest complexity. Heuristic programming in this case is essential. The heuristics developed to make this approach practical range from such immediately necessary constraints as requiring that valence electrons may not exceed the valence shell's capacity and that atoms may not appear in uncharacteristic oxidation states, to the special rules for selecting pathways based on heuristically calculated reaction enthalpies used in the EROS program.[13] The chemist initiating a synthesis search by one of these programs is encouraged to further restrict the scope of the analysis by specifying which bonds in the target molecule are to be synthesized and, since this approach deals with ensembles of molecules, by specifying the complete set of by-product molecules, as well. Further economies are realized in the EROS program by reducing the mechanistic reaction library to just three transforms - which nonetheless span more than 90% of all commonly used synthetic transformations. A recent paper[14] describes facilities for developing synthesis plans in the AHMOS program. In situations where the discovery of new routes to relatively simple, commercially important organic chemicals is desirable, even if this requires finding suitable reagents and conditions for realizing transformations without chemical precedent, this approach shows great promise.

Another approach of interest is that described by Hendrickson[15] as "self-consistent" synthesis planning, wherein the chemical knowledge is available in the form of an abstract representation of all possible carbon-carbon bond construction reactions. This idealistic approach is based on the inherently economical notion that the only synthesis pathways that should be considered for a given molecule are those in which only carbon-carbon bond constructions appear. The most valuable heuristic used in this approach is the concept of a "bond-set" as a strategic, synthesis planning device. In theory, one can analyze the carbon skeleton of any target molecule in terms of a small set of "available" carbon skeletons. For any such set, there are a number of carbon-carbon bonds that must be constructed to give the target molecule's skeleton - these are the bond set. The process of gathering possible synthesis pathways, then, is divided into the task of searching for appropriate sequences of construction

reactions to realize these plans. Another recently described[16] and quite powerful heuristic device is the notion of synthetic distance between the compounds. This is particularly useful for guiding the synthesis search from the target toward one specific available precursor. The extension of this idea to a function that gives a minimum distance from each intermediate to a set of available compounds has also been described.[17]

All the remaining approaches to organic synthesis route discovery utilize reaction libraries built from descriptions of much more specific kinds of chemical transformations. The mixture of specificity and generality that characterizes these libraries is a compromise between the highly skilled labor required to accurately describe the bounds within which application of a transform is known to be appropriate and the surplus of inappropriate pathways generated by overly generalized transforms. The limited nature of the resources available for most of these efforts dictates that a choice must be made between breadth and accuracy.[18] In all cases, there is reliance on the fact that the ability to perceive "good" applications of chemical transforms will operate as an effective heuristic for combating the tendency to exponential growth in the number of possible pathways. The cumulative nature of chemical knowledge bases implies that program performance will continue to improve with time.

A program described by Whitlock and Blower,[19] designed to operated in the rather restricted environment of acyclic organic molecules containing no more than five functional groups, has nonetheless led to the elucidation of some interesting problems in tactics such as that of switching functional groups.[20]

A program first described in 1972 by Bersohn,[21] written in a list processing language called LISP, has since been rewritten in IBM assembly language for the IBM model 370/165 computer to maximize execution efficiency.[4] By developing very efficient algorithms for calculating canonical linear representations[22] and for perceiving rings and functional groups, which, respectively, account for 40, 25 and 10 percent of the total usage of the central processing unit during execution of the program, precursor molecules can be generated at the impressive rate of 14,000 per CPU minute. It should be emphasized here that improvements in algorithmic efficiency, while important, can result in only linearly proportioned decreases in cost, which do not affect the basically exponential costs of the problem. At the rate at which this program generates possible precursors, it is capable of producing many more potential synthesis pathways than even the most dedicated chemist would care to wade through, and so, a variety of heuristic, "tree-pruning" devices have been developed for it. Literature values for yields are stored with each transform in the reaction library,[23] and these values are propagated along the pathway currently being explored. When the cumulative yield for that pathway falls below a preset minimum value, that pathway is rejected, and another is pursued. No record is kept of previously explored pathways. The search method is a preordered, depth-first tree traversal - preordered because the transforms are stored, retrieved and applied in an order expressing the preferences of the chemists responsible for the design of the reaction library. While there is clearly justification for choosing certain powerful transforms first, this method tends not to simulate many actual syntheses where several of the last steps are low-priority functional group interchange reactions, the last synthetic steps being the first to be explored in retrosynthetic analysis. The condition for successful completion of a synthesis pathway is the production of a sufficiently "simple" precursor molecule, where the criteria for determining simplicity are specified at the

time the problem is submitted to the program. One may also specify a single starting material which, when superimposed onto the target molecule, defines which parts of the molecule can be transformed and which cannot.

In the SYNCHEM program,[24] use of application-specific perceptions of chemical transforms is combined with an estimate of the synthetic complexity of each precursor to simulate in a crude way the chemist's almost intuitive feeling that some compounds are simpler to synthesize than others. This combination of knowledge-based estimates of the comparative value of the explored portions of potential synthesis pathways, with an educated guess at the value of the unexplored portion of each pathway, has proved to be powerful for focusing the program's attention on the current most likely pathway. The latter heuristic device, called "compound merit evaluation," has been a functioning component of the SYNCHEM program since its inception,[25] as has its library of available precursor compounds. Another feature of the SYNCHEM program that distinguishes it from the others is its ability to examine the state of the entire problem-solving tree as it grows and to choose paths for further exploration on the basis of this global knowledge about its own progress. Thus, when faced with several equally likely possibilities, SYNCHEM has the option of partitioning its time so that each may be explored essentially in parallel with the others. In the design of SYNCHEM, whenever it was necessary to choose between maximum efficiency of execution and preservation of a maximum of algorithmic generality and flexibility, the latter was always selected. The SYNCHEM reaction library, unlike that described by Bersohn,[23] is composed of pairs of patterns (stored in the form of connection tables) that serve to define suitable substructures which must be present in the target molecule before the transform is selected and also to define the transformation process itself. An improved, more general chemical transformation program module has been described[26] and is part of the current version of this program.

The LHASA program[27] and the SECS program[28] were each originally designed to be used exclusively in the chemist-assisted mode, although each can, at least in principle, suggest (direct) synthesis pathways unaided. In both of these programs, the chemist may select a "strategy" at each point in the interactive decision-making process. These choices directly influence the number and kinds of precursors that will be generated for the next compound selected for further investigation by the chemist in charge. In LHASA, the choices range from the "opportunistic" strategy employed by many of the computer-directed programs, i.e., selecting transforms based on the functional groups that appear in the molecule and generating all possible precursors from them, to the strategy of applying "supertransforms." These supertransforms are capable of producing unaided, moderately long sequences of functional group addition and interchange reactions in order to reach the "subgoal" defined by the chemist. Such subgoals may include the disconnection of a "strategic bond" in a polycyclic bridged molecule[29] or the application of a particularly useful synthetic reaction, such as the Robinson annulation.[30] Some of the chemical concepts developed for implementing these tactical mechanisms, such as that of strategic bonds, can be useful in developing global heuristics for search guidance, much as Hendrickson's bond sets are used. In LHASA these tactics are part of the reaction library data base, described and translated into computer-readable form via the special chemical high-level computer language called CHMTRN. This language, as with other sophisticated languages, suffers the disadvantage of being inaccessible to all without special training in computer programming. The library of CHMTRN descriptions of chemical transformations is translated into an internal code

that is stored with the LHASA program and must be physically in the machine's main memory[31] at all times during the execution of the program. This constraint will eventually limit the size to which the reaction library can grow. Other recent extensions to the LHASA reaction library include a thorough survey of synthetic olefin forming reactions[32] and an in-depth exploration of functional group manipulations involving the very useful halolactonization reaction.[33]

The SECS program[6] also uses a special chemical computer language, called ALCHEM, to describe chemical transformations, but it differs from CHMTRN in two important respects. First, ALCHM is entirely descriptive - it does not interleave instructions for implementing selection heuristics with the information necessary to the transformation process. Second, although ALCHEM is also translated into a machine-readable code, this data is not stored as part of the SECS program. Rather, it is stored on a fast-access magnetic disk and is retrieved in small units as required by the program. The SECS program has served as the prototype for another program called PASCOP[34] that specializes in organophosphorus chemistry. Various versions of the SECS program have been made available to a number of user communities and have been evaluated by them. Gund has commented in detail[35] about experiences with the use of this program in a pharmaceutical research environment, including a discussion of problems with and procedures for encoding chemical information in the ALCHEM language.

Problems and Goals - The first phase in the development of today's organic synthesis discovery programs was characterized by feasibility studies. The second phase involved serious design and implementation of the program modules to do the job. The end of this phase, when all the components were finally assembled and working together, was marked by a certain euphoria - the way seemed clear to the full realization of the original design goals. The third phase began as it became clear that there were limitations that would require new heuristics and, in some cases, extensions to the chemical knowledge base. During this third phase, growth in the size of the chemical knowledge base has tended to be incremental rather than geometrical, program reliability has generally tended to increase, and new, more subtle programs have manifested themselves.

System maintenance and reliability have become non-trivial problems for such inevitably large and complex programs. Fortunately, the body of conventional computer scientific wisdom on such matters as program documentation, modular design, top-down structured programming, plus rigid adherence to organizational protocol, is sufficient to keep things manageable for even the largest of these programs.

One factor that will be important for the continued reliability of these programs is the choice of programming language. The PL/1 language, in which the SYNCHEM and EROS programs have been written, is one of the best general-purpose information processing languages available today and is most suitable for writing well-structured large computer programs. Its debugging facilities, an important factor in program maintenance, are very good. However, PL/1 compilers are not available for all types of computers currently in use, and so programs written in this language are largely restricted for the moment to installations with IBM equipment. The FORTRAN language, while older and much more primitive in its capabilities, has the distinct advantage of running with only minor modifications on most computers. This is the language in which the LHASA and SECS programs have been written. As Bersohn has shown, it is possible to make great gains in efficiency by programming in the assembly language of a partic-

ular computer. The price paid in this case, however, is complete lack of program portability, as well as greatly increased maintenance problems.

Organization and management of the chemical knowledge base is another potentially serious problem, as is the related problem of acquiring additional information for it. When the amount of data is small, in the early stages in the development of the knowledge base, the tendency is not to be greatly concerned about organization and to concentrate on a machine-readable representation. As the amount of data grows, organization and management remain essentially an external human task. At some point, the amount of data and the cost in time spent managing it is so large that there is no alternative but to transfer all management functions to the computer. This may mean designing a much more elaborate data storage and retrieval system than was originally considered. Part of this facility for storage and retrieval must be a capability for translating information readily from an internal, compact, machine-readable format into an external, human-readable format and back. The main problem here is the human to machine translation. Humans do not enjoy being restricted to expressing their ideas in terms of an artificial highly structured language suitable for easy machine translation, and machines are not particularly efficient yet in understanding natural human languages, or even the subsets of such languages that make up the jargons of the sciences. Whether the format is an English-like computer language, such as ALCHEM, or an efficiently translatable transform description using pairs of connection tables, most potential contributors to a chemical knowledge base are put off by the artificiality of the medium of communication. One proposal for dealing with this problem is to interpose a trained human translator between the committee of contributors and the computer. Another solution, which has met with some initial success, is to have the computer engage the contributing chemist in a dialogue[36] wherein a natural language interchange can be approximated. The computer can extract the required information from such a dialogue and cast it into the required format without the contributor having to concern himself with any non-chemical details. Yet another proposal[37] is to train the computer to read the chemical literature directly. The problem of extracting the required generalized transforms from a series of specific examples, i.e., the simulation of an inductive thought process, is itself a very interesting and unsolved problem in artificial intelligence.

The original, central problem of developing efficient strategies for finding valid, sensible, "good" synthesis pathways still awaits a complete solution - if one exists. This is a problem for which chemists must provide the evaluation criteria. Much has already been done in this direction, to the benefit of practicing chemists, as much as to the benefit of the designers of organic synthesis discovery programs. The methods eventually developed to enable the computer to "intelligently" solve such difficult and important problems may bear little resemblance to the ways that chemists solve these problems.

Conclusion - Computer-directed organic synthesis route discovery has advanced to the point where a number of groups in the pharmaceutical industry and in other chemically-oriented industries are considering its potential as a sophisticated tool for the retrieval of chemical information and for directly aiding the organic chemist in planning and evaluating possible syntheses of important compounds. Among the advantages accruing to such usage is that it is an effective means of circumventing the natural biases of individual chemists responsible for designing syntheses. Putting practical applications aside, the existing organic synthesis programs

are proving to be suitable vehicles for testing hypotheses about synthesis planning strategies, stimulating more thinking about synthesis planning in the abstract and about exactly what the criteria should be for deciding what constitutes a good synthesis. The body of rules and methods already proposed for synthesis planning has had in turn a significant impact on the teaching of this subject in graduate schools. The next generation of synthetic organic chemists will look on the development of new synthesis routes not so much as an art but more as a science - although there will always be art in what is done well!

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Chapter 26. Recent Progress in the Design of Suicide Enzyme Inhibitors

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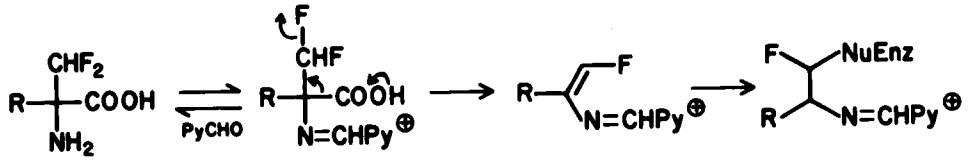
An attractive approach to the irreversible inactivation of enzymes is the use of inhibitors possessing latent reactive functionalities which are unmasked at the enzyme's active site. Since the enzyme becomes inactivated as a consequence of its own mechanism of action, such inhibitors have been referred to as "suicide inhibitors."[1] An inhibitor acting by such a mechanism would be expected to be extremely specific, because it should inactivate only those enzymes for which it is a substrate. It could also be expected to exhibit long-lasting biochemical and pharmacological effects *in vivo*, as the enzyme should remain inactivated even after free inhibitor is no longer present. Regeneration of enzyme activity would depend on new enzyme synthesis. The reader is referred to reviews by Rando,[2] Abeles and Maycock[1] and Walsh,[3] as well as to the proceedings of two symposia,[4,5] which offer comprehensive reviews of the area up to 1978. This report will attempt to summarize significant findings, since the previous Annual Report chapter on enzyme inhibition by Jung.[6]

A number of α-difluoromethyl and α-monofluoromethylamino acids are potent irreversible inhibitors of the corresponding *amino acid decarboxylases*.[7,8] The proposed general mechanism of inhibition, illustrated for α-difluoromethylamino acids, involves enzyme-induced decarboxylation of the pyridoxal phosphate (PyCHO)-derived Schiff base, leading to elimination of fluoride ion and generation of an alkylating agent in the active site.



Ornithine Decarboxylase - This rate limiting enzyme in the biosynthesis of polyamines was previously reported to be inactivated *in vitro* by α-difluoromethylornithine (1).[7,9] This compound has now been found to have antigestational,[10] antitrypanosomal,[11] anticoccidial[12] and antitumor[13] activities *in vivo*, thus underlining the antiproliferative effects of polyamine depletion. α-Monofluoromethylornithine (2)[8] and α-acetylenic-ornithine (3)[14] have been reported to have enzyme inhibitory activity similar to 1 *in vitro*. It is believed that 3 inactivates via an enzyme-catalyzed decarboxylation, leading to the formation of a conjugated allene which, being a Michael acceptor, can trap a nucleophilic residue in the active site.[14]

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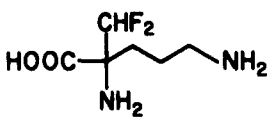

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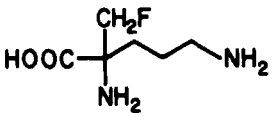

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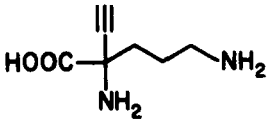
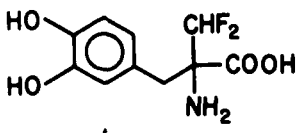


3

Aromatic Amino Acid Decarboxylase - This enzyme, which is responsible for the conversion of dopa to dopamine, is subject to inactivation by α-difluoromethyldopa (4)[15] and by α-monofluoromethyldopa (5).[16,17] The former compound has a selective peripheral action and, hence, increases central dopa, dopamine and noradrenalin levels.[15] In contrast, 5, a more potent inactivator, inhibits both central and peripheral activities, depletes central catecholamine levels and has antihypertensive properties.[16,17]



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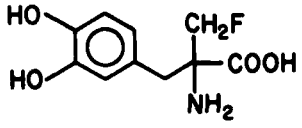

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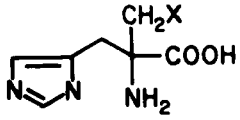
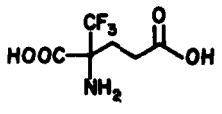


6 X=Cl

7 X=F

Histidine Decarboxylase - This enzyme, which catalyzes the conversion of histidine to histamine, is irreversibly inactivated by both α-monochloromethylhistidine (6)[18] and α-monofluoromethylhistidine (7).[8] Both inhibitors are effective in vivo, and 6 has been reported to inhibit gastric acid secretion.[18] Compound 7 is the more potent inhibitor, and its effect is longer lasting,[19] presumably due to the instability of 6.

Glutamic Acid Decarboxylase - Three suicide inactivators, 8,[20] 9 and 10,[21] of this enzyme, which catalyzes the formation of the inhibitory neurotransmitter GABA, have been recently reported. While 8 and 9 presumably inactivate by the foregoing general mechanism, 10 is envisaged to undergo enzymatic decarboxylation with direct formation of a Michael acceptor. No in vivo data have been reported for these compounds. So far, the only suicide inactivator of mammalian glutamic acid decarboxylase reported to be active in vivo is (S)-4-aminohex-5-ynoic acid (11);[22] which also inactivates GABA-transaminase.



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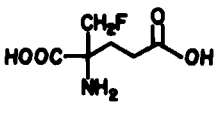

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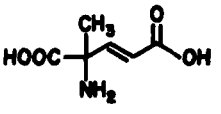

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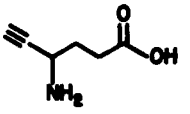
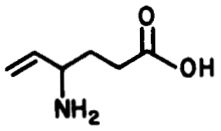


11

GABA Transaminase - Inactivation of this enzyme blocks GABA catabolism and, thereby, increases the availability of that neurotransmitter in the CNS. Several inactivators have been described,[23] one of which, γ-vinyl GABA (12), has been found to increase GABA levels in human cerebrospinal fluid in a dose-dependent manner [24] and is undergoing clinical trial in

several neurological disorders. Although trans-4-aminocrotonic acid (13) is an excellent substrate for GABA-T and could be expected to be transformed to a potential alkylating agent, no inactivation takes place. Incorporation of halogen atoms on the double bond, as in 14[25] and 15,[26] results in irreversible inhibition. Evidently, in these cases, the electrophilicity of the enzyme-generated alkylating agents is increased.



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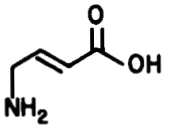

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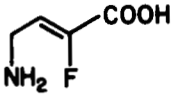

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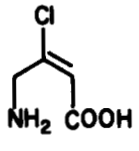
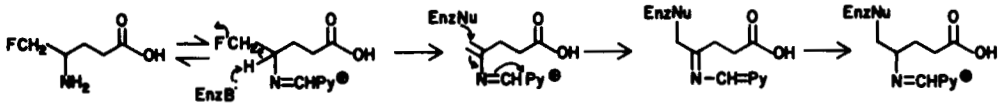


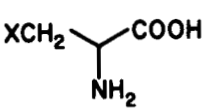
15

Recently, 4-amino-5-fluoropentanoic acid (16) has been reported to inactivate GABA-T.[27] The proposed mechanism involves enzymatic proton abstraction, β-elimination of fluoride and generation of a reactive alkylating agent. In vivo data for 14, 15 and 16 are not yet available.



16

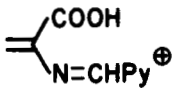
Alanine Racemase - The D isomers of β-chloro and β-fluoroalanine (17 and 18) are known to inactivate alanine racemase, an enzyme necessary for bacterial cell wall synthesis. Recent studies on these and on O-carbamoyl-D-serine (19) and their corresponding L-isomers have shown that the ratio between enzymatic conversion to pyruvate and inactivation is approximately 830:1, regardless of Vmax.[28] This suggests that a common intermediate, presumably the aminoacrylate-PyCHO complex (20), is responsible for inactivation.



17 X=Cl

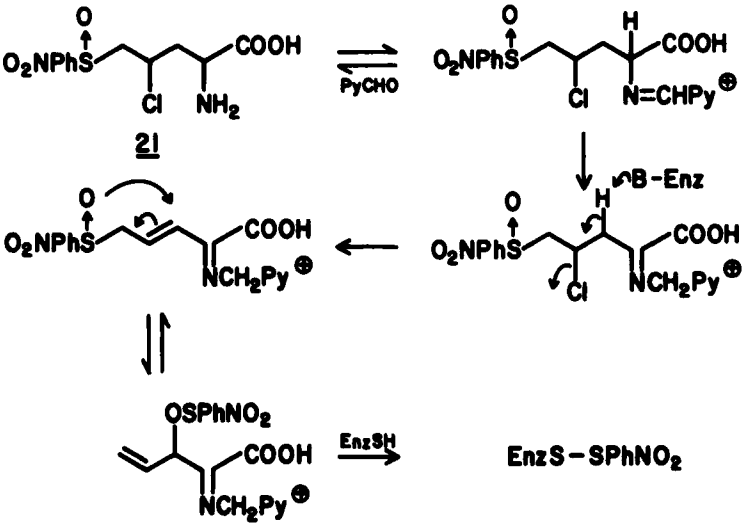
18 X=F

19 X=$OCONH_2$

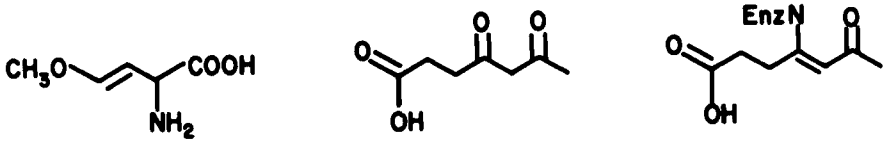


20

The mechanism of each of the inactivators described above relies on enzymatic transformation to a Michael acceptor, which then alkylates some nucleophilic residue in the active site. A novel form of suicide inactivation has been devised, which is based on the formation of an allyl sulfenate by a 2,3 sigmatropic shift of an allyl sulfoxide generated enzymatically via a β-elimination process. Thus, 21 inactivates cystathionine γ-synthetase and methionine γ-lyase by the mechanism shown.[29]



The rate-limiting enzyme of heme biosynthesis, δ-aminolevulinic acid (ALA) synthetase is inactivated, apparently in a suicidal manner, by 22.[30] Presumably 22, an analog of the normal substrate glycine, becomes transaminated to generate a Michael acceptor in a similar manner to that found operative for the inactivation of aspartate aminotransferase by this compound.[31]

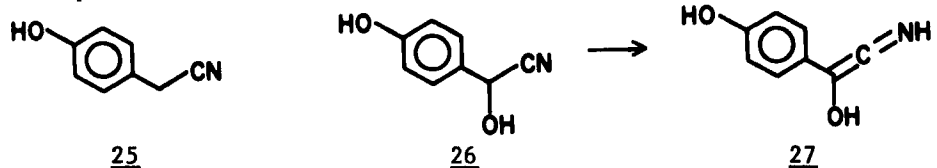


22 23 24

ALA Dehydrase - This enzyme, which condenses two molecules of ALA via a lysine Schiff base intermediate to form porphobilinogen, is inactivated by the β-diketone, 4,6 dioxoheptanoic acid (23).[32] The mechanism may involve formation of the stable vinylogous amide 24.

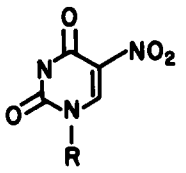
The mechanism by which cyclopropyl amines inactivate monoamine oxidase has been studied and has been found to be of the suicide type,[33,34] the cyclopropylamine moiety being converted to cyclopropylimine as part of the inactivation process. Cytochrome P-450 is also inactivated by cyclopropylamines,[53] presumably via a similar mechanism.

Dopamine β-Hydroxylase - A copper-containing enzyme dependent on oxygen and ascorbate, dopamine β-hydroxylase is inactivated by p-hydroxybenzyl cyanide (25), which undergoes conversion to p-hydroxymandelonitrile (26). The inactivation may proceed via an enzymatically-induced prototropic shift leading to the ketenimine 27, which could alkylate some enzyme nucleophile.[35]



25 26 27

Thymidilate Synthetase - This enzyme has attracted attention as a target for inhibitors with potential chemotherapeutic utility as a result of the activity of fluorodeoxyuridine monophosphate formed in vivo from 5-fluorouracil. Recently described inactivators are 5-nitro-2'-deoxyuridilate (28),[36,37] 5-ethynyl-2'-deoxyuridilate (29)[38,39] and trans-5-(3,3,3-trifluoro-1-propenyl)-2'-deoxyuridylate (30).[40] An early event in the



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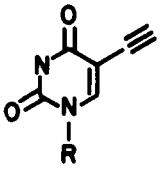

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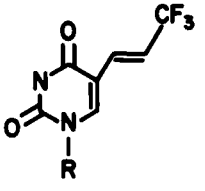

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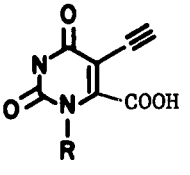
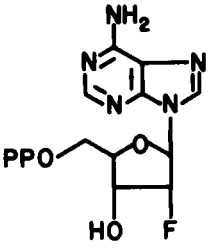


31

normal catalytic mechanism of this enzyme involves nucleophilic attack by an enzyme SH at the 6 position of deoxyuridine monophosphate, and this also occurs with 28, 29 and 30. In the case of 29 and 30, such a nucleophilic attack would lead to the formation of a Michael acceptor at position 5 (allene from 29, difluorobutadiene from 30), while with 28 a second alkylation event does not occur.

5-Ethynylorotic acid (31) has been reported to be an irreversible inhibitor of dihydroorotate dehydrogenase.[41] Presumably, on reduction, 31 affords a conjugated allene, which alkylates an active site nucleophile.

Ribonucleotide Reductase - This enzyme catalyzes the reduction of ribonucleoside 5'-diphosphates to the corresponding 2'-deoxyribonucleoside 5'-diphosphates and, hence, plays a critical role in the de novo synthesis of DNA. 2'-Deoxy-2'-fluoroadenosine 5'-diphosphate (32) is an inactivator of the enzyme, the inactivation event being accompanied by liberation of fluoride, inorganic pyrophosphate and base. It has been suggested that the enzyme-generated inactivating species is the Michael acceptor 33.[42]



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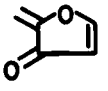
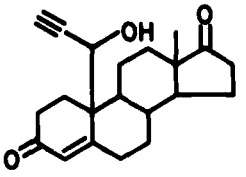


33

Aromatase - A cytochrome P-450 dependent enzyme, aromatase, which catalyzes the conversion of testosterone to estradiol, is inactivated by the propargylic alcohol 34.[43] This compound was designed to be hydroxylated at C-19 in the same manner as the normal substrate. It was hoped that in situ dehydration of the resultant gem-diol would then lead to the formation of the acetylenic ketone 35, capable of covalently modifying the enzyme.

Cytochrome P-450 is involved in the hepatic metabolism of lipophilic substances. The mechanism of cytochrome P-450-mediated hydroxylation of unactivated C-H bonds and epoxidation of olefins is thought to be an oxygen atom insertion process from a heme-oxene complex. Ortiz de Montellano and collaborators have elucidated the mechanism of

inactivation of the inducible hepatic enzyme by various acetylenes[51] and allenes.[52] In these cases, oxygen insertion into the π bond generates unstable unsaturated epoxides or allene oxides, which could alkylate the heme prosthetic group. Such a mechanism could also explain the inactivation of aromatase by compound 34.



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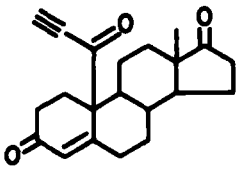
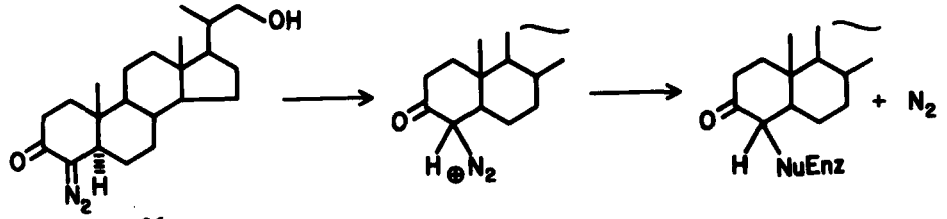


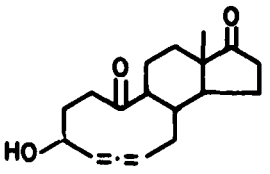
35

Testosterone 5-α-Reductase - NADPH dependent testosterone 5-α-reductase catalyzes the reduction of testosterone to the more potent androgen, dihydrotestosterone. The enzyme is inactivated by the diazoketone 36.[44] It has been proposed that the protonation step, implicit in the mechanism of reduction of testosterone, activates the diazoketone function, which then alkylates an active site nucleophile.



36

3α-Hydroxysteroid Dehydrogenase - This enzyme is also NADPH dependent and is inactivated by the secosteroid 37, which is enzymatically converted to the corresponding allenic ketone, an alkylating agent.[45] A similar mechanism applies in the inactivation of 3α, 20β-hydroxysteroid dehydrogenase by the propargylic alcohol 38.[46] Interestingly, the diazoketone 36 does not inactivate a similar enzyme from rat prostate.[44]



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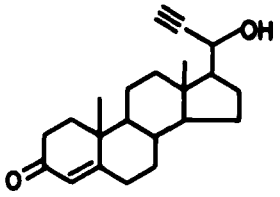
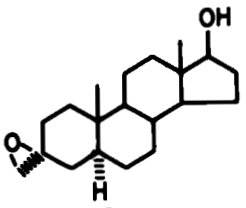


38

Δ5-3-Ketosteroid Isomerase - This enzyme, which catalyzes the conversion of Δ5,6-3 ketosteroids to their Δ4,5 isomers via a proton abstraction-reprotonation mechanism, is inactivated by the epoxide 39.[50] The same active site moieties, which effect enolization of the normal substrate, should effect protonation of the β-epoxide, thus facilitating nucleophilic attack by an active site nucleophile. Although the α-epoxide is a competitive inhibitor, no inactivation occurs.

Allyl alcohol inactivates yeast alcohol dehydrogenase via conversion to acrolein, which diffuses from the active site and then inhibits the enzyme by an affinity labelling mode.[47] It is a poor inhibitor of the liver enzyme. If a leaving group is placed on the double bond as in 3-ethylthioprop-2-en-1-ol (40), the oxidation product is

apparently trapped before it diffuses from the active site.[48] In an extension of this work, it has been found that the cyclopropyl carbinol 41 also inactivates the liver dehydrogenase, the major pathway for inactivation probably being oxidation to the aldehyde followed by a homo-Michael addition of enzyme nucleophile.[49]



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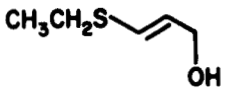

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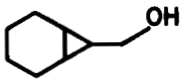
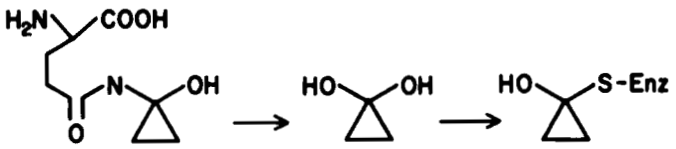


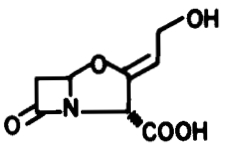
41

Cyclopropanone formation is the mechanism by which coprine (42) inactivates aldehyde dehydrogenase in vivo.[54] Thus, hydrolysis of 42 leads to the liberation of cyclopropanone hydrate. Aldehyde dehydrogenase catalyzes the dehydration of the hydrate to cyclopropanone, which then forms a kinetically stable thiohemiketal with the active site thiol. This thiohemiketal would be analogous to the thiohemiacetal thought to be an obligatory intermediate in the enzyme catalyzed oxidation of aldehydes.



42

β-Lactamases - The β-lactam ring of penicillins and cephalosporins is hydrolyzed by β-lactamases, making the organism resistant to these substances. Recently, it has been found[55] that clavulanic acid (43)[56] and the penicillanic sulfone 44[57] are suicide inactivators of these enzymes. It is proposed that an initial acyl enzyme complex fragments via a β-elimination process wherein enolate and sulfinate are the respective leaving groups. The remaining acyl-bond is stabilized to hydrolysis by the unsaturation of C5-C6 as in 46.[56] Apparently, the 6-α-chloropenicillanic sulfone 45 inactivates in a similar manner.[58]



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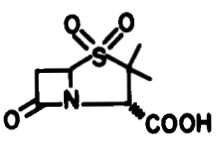

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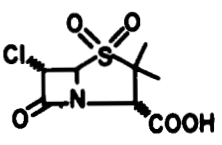

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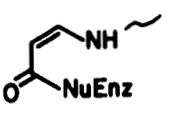
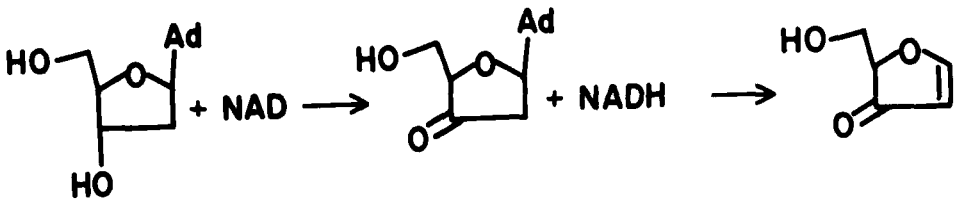


46

Although each of the suicide inactivators discussed in this chapter owes the irreversibility of its action to covalent bond formation, this is not a prerequisite for irreversibility, as demonstrated earlier for the GABA-T inactivators gabaculine[59] and isogabaculine.[60] It appears

that the inactivation of 5-adenosyl-homocysteine hydrolase by 2'-deoxy-adenosine 47[61] is another example which may not involve covalent bonding. The normal enzymatic mechanism involves a reversible reduction of enzyme bound NAD to NADH and oxidation of the 3'-OH group of the substrate.[62] It has been proposed that the inactivation process induced by 47 involves irreversible reduction of the NAD.[63] The oxidation product from 47, without the 2'-hydroxy group, is unstable and fragments leaving the enzyme in the reduced form, which cannot bind substrate nor revert to the oxidized form.



47

Conclusion - Since the appearance of the previous chapter,[6] the field of suicide inhibition has advanced with several of the inactivators described previously undergoing clinical development, while conceptual advances have led to broadening the approach to additional classes of enzymes. Suicide inhibition 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 and will remain a powerful option.

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Chapter 27. Sequencing and Synthesis of Nucleic Acids

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Introduction and Overview - Genes and gene control regions can now be routinely characterized and studied at the molecular level. This is possible because of several recent advances in the technology associated with manipulating and modifying DNA sequences. Perhaps the first major advance in this area was the development of bacterial plasmids as vehicles for introducing nonbacterial genes into bacteria.[1] Using these molecular cloning techniques, very pure and greatly amplified mammalian genes became available for the first time.[2,3] A parallel and complementary development was the discovery of the host restriction modification enzymes.[4,5,6] These endonucleases recognize unique DNA sequences (usually four to six nucleotide base pairs) and cleave both DNA strands at sites usually within this sequence.[7] As a result of this recognition specificity, these enzymes can be used to precisely order DNA sequences relative to one another and consequently generate a map of overlapping DNA segments representing several thousand base pairs or in some cases even total genomes.[8-13]

A more recent technical advance has been the development of techniques for determining DNA sequences. Only nine years ago, the longest known DNA sequence was a 20-base pyrimidine tract from the filamentous coliphage fd.[14] Achievements now range to the sequence of total chromosomes[11-13] and are measured in tens of kilobases; a major problem in this area now involves applying computers to store and analyze DNA sequence information.[15] Finally, recent progress in nucleic acid chemistry has led to the routine synthesis of deoxyoligonucleotides containing 8 to 20 mononucleotides.[16,17] These synthetic DNAs have been used as probes to locate genes[18] and as intermediates in the total synthesis of genes.[19-23] As a result of these important advances in DNA technology, the purification, amplification, and expression of medicinally useful genes and gene systems is now possible. This review will emphasize recent advances in these latter areas, the sequencing and synthesis of DNA.

Nucleic Acid Sequencing - There are two basic approaches for sequencing DNA. The methodology has been published in considerable detail.[24,25] The objective for each approach is to generate a series of deoxyoligonucleotides from the DNA segment of unknown sequence. All the deoxyoligonucleotides would have one common, radioactively labeled end, but would vary in

$$d(\overset{*}{p}ApGpCpGpTpG) \rightarrow d(\overset{*}{p}A) + d(\overset{*}{p}ApG) + d(\overset{*}{p}ApGpC) + d(\overset{*}{p}ApGpCpG) + d(\overset{*}{p}ApGpCpGpT) + d(\overset{*}{p}ApGpCpGpTpG)$$

Figure 1. The symbols A, G, C, T represent deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxythymidine respectively; p represents the internucleotide phosphate and $\overset{*}{p}$, a [^{32}P] labeled phosphate.

length at the other by one nucleotide (Fig. 1). This strategy assures that all possible homologs are present. These deoxyoligonucleotides are

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then ordered as to size on polyacrylamide gels using high voltage electrophoresis and identified. The approaches differ by the methodology used to generate the homologous series of deoxyoligonucleotides. Maxam and Gilbert have developed a series of chemical reagents that are base-specific,[26] while Sanger has used an enzymatic method.[27]

Sequencing DNA using the base specific chemical cleavage approach involves three steps: modification of a base, removal of the modified base from its sugar, and DNA strand scission at that sugar. Consider for example the specific removal and identification of guanine residues. Dimethyl sulfate alkylates nondenatured DNA primarily on the N-7 of the guanine and the N-3 of adenine. By carefully controlling the reaction conditions, essentially exclusive methylation will occur on guanine.

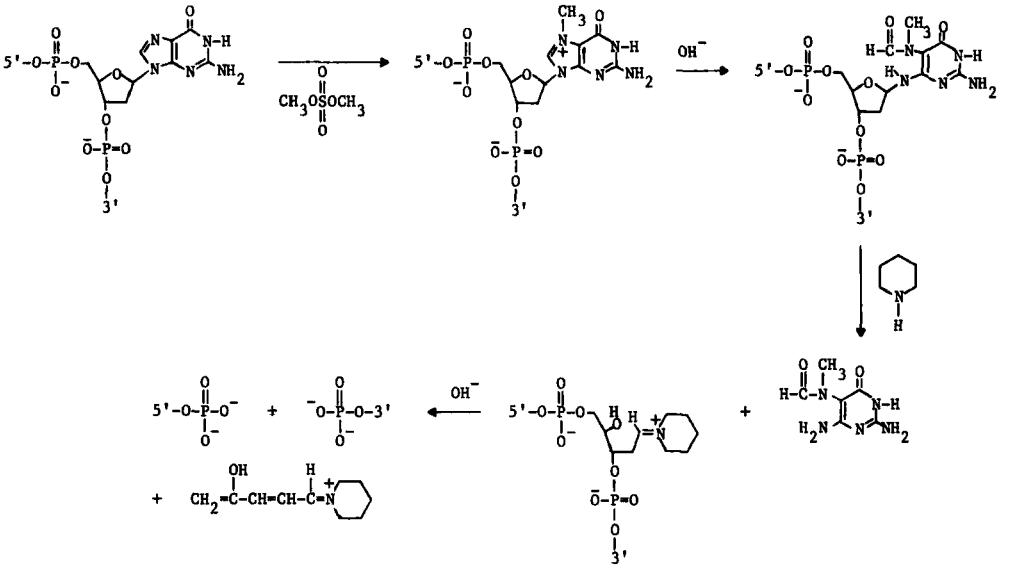

Figure 2. Reaction of Dimethyl sulfate/Piperidine with Guanosine.

Further treatment with alkali and piperidine leads initially to ring opening, displacement of the base by piperidine, and finally base catalyzed β-elimination leading to cleavage of the DNA chain (Fig. 2). In order to use this chemistry for DNA sequencing, reaction conditions must be chosen so that alkylation at any one guanine is incomplete, but subsequent steps (base removal and β-elimination) go to completion at alkylated sites. As an example, consider the previously described hexanucleotide radioactively labeled with a [5'-^{32}P] phosphate. After treatment with dimethyl sulfate and aqueous piperidine, three labeled deoxyoligonucleotides would be

$$d(\overset{*}{p}ApGpCpGpTpG) \xrightarrow[2.\ \text{Aqueous piperidine}]{1.\ \underline{\text{Dimethyl sulfate}}} d(\overset{*}{p}Ap) + d(pApGpCp) + d(\overset{*}{p}ApGpCpGpTp)$$

Figure 3. Abbreviations are outlined in Fig. 1.

produced, if alkylation on any one guanine were incomplete (Fig. 3). These partial degradation products can be separated by gel electrophoresis

and will appear as a series of radioactively labeled bands. Thus, with a large deoxyoligonucleotide, a ladder of bands would be present. Each band indicates the position of a guanine mononucleotide in the overall sequence. A complementary chemical reaction involves using piperdine formate (pH 2.0) to cleave DNA (again partially) at all purine sites (adenine and guanine). Thus, on a slab gel, a ladder of bands would be observed indicating positions of mononucleotides that were either guanine or adenine. By analyzing the dimethyl sulfate reaction product (guanine only) and the piperidine formate product (adenine and guanine) side-by-side on the same slab gel, unique positions containing either guanine or adenine can be determined. Similarly, hydrazine has been shown to react with thymine and cytosine bases and generate deoxyribosyl urea at pyrimidine sites within the deoxyribose phosphate backbone of a DNA polymer. β-Elimination catalyzed by aqueous piperidine then generates a DNA free of all pyrimidines. If molar sodium chloride is present during this hydrazine reaction, only cytosine reacts appreciably. Therefore by limiting the extent of reaction with hydrazine and by varying the ionic strength, thymine and cytosine mononucleotides can be differentiated by slab gel electrophoresis in a manner completely analogous to the method used to differentiate the purines. The diagram shown in Figure 4 outlines an idealized slab gel electrophoresis pattern expected for the hexanucleotide whose sequence is shown in Figure 1. This method is extremely fast and can be used to

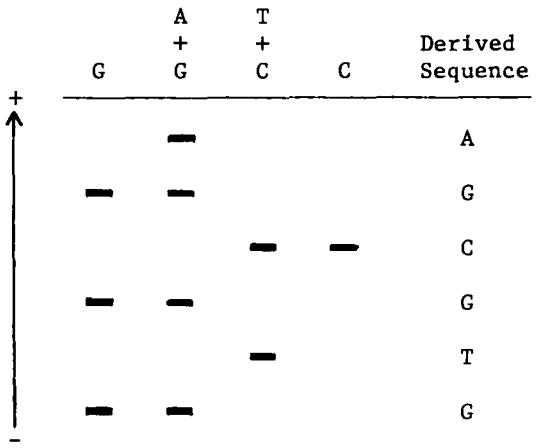

Figure 4. Gel Electrophoresis of Partially Degraded DNA Samples. Samples marked G, A + G, T + C, and C designate d(p*ApGpCpGpTpG) treated with dimethyl sulfate, piperidine formate, hydrazine, and hydrazine containing sodium chloride, respectively.

sequence deoxyoligonucleotides containing approximately 100 to 200 mononucleotides per experiment. By using overlapping DNA segments generated by cleavage with different restriction enzymes, thousands of contiguous base pairs can be sequenced using this methodology.

The enzymatic sequencing approach (chain terminator method) is based on the ability of DNA polymerases to accurately synthesize a complementary radioactive copy of a single stranded DNA template using a primer (usually generated with an appropriate restriction enzyme). The chain terminator method uses the 2', 3'-dideoxy or the β-D-arabinofuranosyl analogs of the

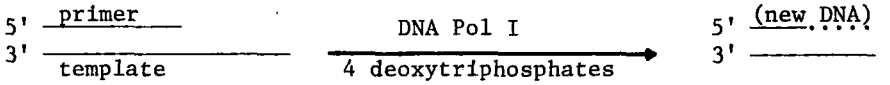

Figure 5. Reaction scheme for preparing Labelled Homologous DNAs.

deoxyribonucleoside triphosphates as a means to sequence DNA. These analogs can be incorporated into DNA but lack a 3'-hydroxyl group. Therefore chain termination occurs whenever one of these analogs is incorporated into DNA. Experimentally, the procedure involves four separate repair reactions containing *E. coli* DNA polymerase I and all four deoxytriphosphates (any one being radioactively labelled). The 2', 3'-dideoxy analog of thymidine 5'-triphosphate is then added to one reaction, the 2', 3'-dideoxy analog of cytidine 5'-triphosphate is added to the second reaction and so on for the other two. There is, therefore, a base-specific partial incorporation of a terminating analog into each position throughout the DNA sequence of interest. These four partially repaired, homologous DNAs are then fractionated as to size by gel electrophoresis. The reaction mixture containing 2', 3'-dideoxythymidine will display a series of bands indicating thymidine positions in the sequence (the incorporation of dideoxythymidine). The other repair reactions will display similar gel patterns indicating positions for cytosine, guanine and adenine. Thus, by comparing the location of bands generated from each reaction mixture, a sequence for the deoxyoligonucleotide can be deduced. This method is also extremely rapid, versatile and easy to use. The two methods have several subtle differences which often dictate which procedure should be used.[24,25] Clearly, the ability to use either method is a distinct advantage for anyone involved in sequencing DNA.

Procedures are also available for sequencing RNA. The most comprehensive method can be used to accurately determine the modified RNA nucleotides, in addition to uracil, guanine, cytosine and adenine.[28] Another very useful RNA sequencing method is very similar to the base specific chemical cleavage approach outlined previously. Enzymes, rather than chemical reagents, are used to cleave RNA at specific sites.[29]

Nucleic Acid Synthesis - A large number of synthetic strategies for preparing sequence defined deoxyoligonucleotides have been developed.[16,17] The major challenge is formation of the internucleotide phosphate ester bonds. A closely related but secondary problem is the developement of amino, phosphorus and hydroxyl blocking groups that are compatible with the formation of internucleotide bonds. As work in this area has progressed, three chemical methodologies have evolved which differ primarily in relation to the synthesis of the internucleotide phosphodiester bond. More specifically, these methods involve the direct synthesis of the phosphodiesters, the intermediate synthesis of phosphotriesters, and the formation of intermediate phosphite triesters. All three methods can be illustrated using the general scheme depicted in Figure 6.

The diester approach involves the condensation of a nucleoside (I: R_1, *p*-anisyldiphenylmethyl; R_4, hydrogen) with a nucleotide (II: R_5, phosphate; R_2, aryl or acyl ester) using an activating agent such as dicyclohexylcarbodiimide or arylsulfonyl chlorides in anhydrous pyridine.[30] The product is an appropriately protected phosphate diester (III: R_3, hydrogen). Protecting groups are removed from III using concentrated ammonium hydroxide (R_2 and base protecting groups) and dilute acetic acid (R_1). Deoxyoligonucleotides as large as eicosanucleotides have been prepared by

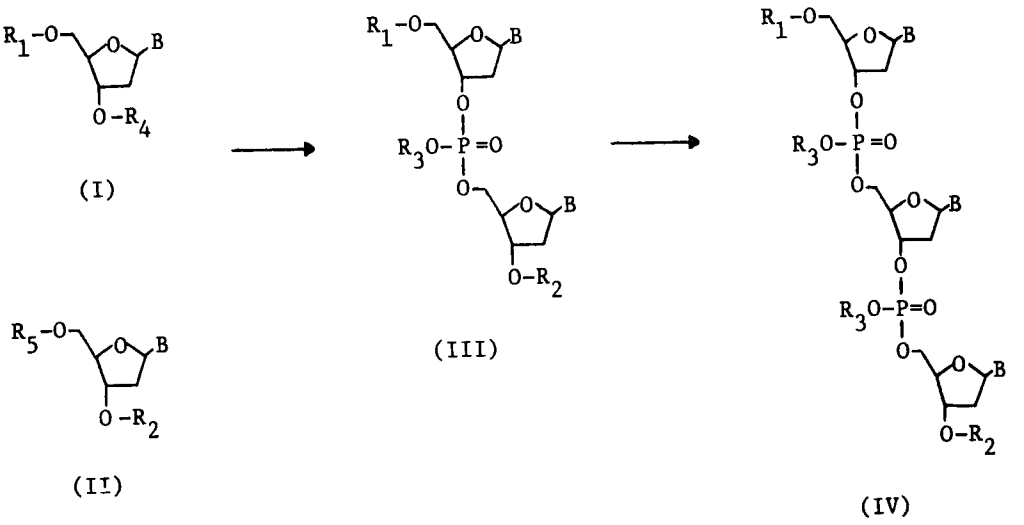

Figure 6. General Scheme for Synthesis of Polynucleotides. B represents a protected purine or pyrimidine base. R groups are defined in the text.

these procedures.[31,32] The synthetic products can be readily purified by diethylaminoethyl cellulose ion exchange chromatography or reverse phase high pressure liquid chromatography. Yields per condensation range from 30% to 60%. This approach has been used to synthesize sequence-defined polynucleotides useful for studies on the genetic code,[33] the synthesis of lactose operator and lambda operator DNAs,[34,35] the synthesis of an alanine transfer RNA gene,[19] and the first total synthesis of a biologically active gene-the *E. coli* tyrosine suppressor III transfer RNA gene.[20] A major advantage of this approach, as contrasted to others, is that mononucleotide starting materials are readily available and can be easily derivatized for polynucleotide synthesis. The major problem inherent in this approach is the activation of unprotected (III, IV: R_3 = H) phosphodiester internucleotide bonds contained within the starting materials and product. This activation by the condensing agent leads to undesirable side reactions which dominate the synthetic procedures as the chain length increases and also reduces the overall yield.[16,17]

The triester approach involves the synthesis of phosphate triesters as intermediates in polynucleotide synthesis. These triesters are then converted to phosphodiesters. The most attractive feature of this approach is that the highly reactive internucleotide phosphate anions are masked as triesters. The approach was initially developed by Letsinger in his work on polymer supports[36-38] and led to the systematic development of a phosphotriester synthetic methodology,[39,40] which in modified forms is now used widely in the nucleic acid field.[16,17] The current most popular synthetic strategy is the following.[41,42] A nucleotide phosphodiester (I: R_1, di-*p*-anisylphenylmethyl; R_4, *p*-chlorophenylphosphate) is condensed with a second nucleotide (II: R_5, hydrogen; R_2, β-cyanoethyl-*p*-chlorophenylphosphate) using an activating agent such as triisopropylbenzenesulfonyltetrazolide (TPSTe). Using the nucleotide phosphodiester I in 100% molar excess, the condensation with TPSTe as a coupling reagent is reported to go essentially to completion. The isolation procedure involves

simply treatment with benzenesulfonic acid to remove the trityl ether (R_1, di-*p*-anisylphenylmethyl) from III, followed by aqueous extraction of starting materials and other ionic components, such as triisopropylbenzenesulfonate. Precipitation of the organic phase gives essentially homogeneous dinucleotide (III). This synthesis procedure can then be repeated a second time using the detritylated dinucleotide (III: R_1, hydrogen; R_2, β-cyanoethyl-*p*-chlorophenylphosphate; R_3, *p*-chlorophenyl) and a mononucleotide (I: R_1, di-*p*-anisylphenylmethyl; R_4, *p*-chlorophenylphosphate). The trinucleotide (IV: R_1, di-*p*-anisylphenylmethyl; R_3, *p*-chlorophenyl; R_2, β-cyanoethyl-*p*-chlorophenylphosphate) is then isolated by short column silica gel chromatography. This trinucleotide is a very versatile reagent for further synthesis. The trityl ether can be removed from one trinucleotide (IV: R_1, hydrogen; R_3, *p*-chlorophenyl; R_2, β-cyanoethyl-*p*-chlorophenylphosphate) and the β-cyanoethyl group can be removed using mild base from another trinucleotide (IV: R_1, di-*p*-anisylphenylmethyl; R_3, *p*-chlorophenyl; R_2, *p*-chlorophenylphosphate). These two trinucleotides can be joined using TPSTe to form a hexanucleotide. Repetition of this cycle with various hexanucleotides will lead to formation of dodecanucleotides. Since reactive groups can be generated at either end of a trinucleotide IV (R_1, hydrogen, or R_2, *p*-chlorophenylphosphate), syntheses can proceed either in a 3'- to 5'- or in a 5'- to 3'- direction. Thus, by synthesizing all 64 possible trinucleotides, the ability to rapidly synthesize any deoxyoligonucleotide is possible. This same basic methodology has been used for synthesizing deoxyoligonucleotides on insoluble polymer supports. Considerable success has been achieved using polyacrylamide,[43] polyacrylmorpholide,[44] cellulose,[45] cross-linked polystyrene,[46] and polystyrene grafted on teflon beads.[47] Generally each synthesis begins by reacting compound I (R_1, di-*p*-anisylphenylmethyl; R_4, hydrogen) with a carboxylic acid functional group located on the polymer. The resulting ester anchors the growing deoxyoligonucleotide to the insoluble polymer support. After removal of the trityl ether, synthesis of the deoxyoligonucleotide proceeds by successively adding di- or trinucleotides using the same chemistry as described above for the nonpolymer support phosphotriester approach. Syntheses, however, proceed only in a 3'- to 5'- direction. Yields per condensation have been reported to be 80% to 90%. After several cycles, the final product is removed from the support with base. Isolation is completed by ion exchange chromatography after removal of protecting groups. Using presynthesized trinucleotides as condensing units and TPSTe as activating agent, deoxyoligonucleotides containing as many as 28 mononucleotides have been synthesized.[46]

A recent innovation in oligonucleotide synthesis has been the introduction of the phosphite coupling approach by Letsinger and coworkers.[48-50] This approach has been adapted to the synthesis of deoxyoligonucleotides,[51-55] oligoribonucleotides,[56-59] and nucleic acid analogs.[60-62] Generally, the approach involves the reaction of a suitably protected nucleoside (I: R_1, di-*p*-anisylphenylmethyl; R_4, hydrogen), a bifunctional phosphitylating agent, such as methoxydichlorophosphine or trichloroethyldichlorophosphine, and a second protected nucleoside (II: R_5, hydrogen; R_2, levulinyl). Mild oxidation using iodine in tetrahydrofuran, lutidine and water generates the natural internucleotide bond. By varying the oxidation procedure, phosphorus analogs, such as selenophosphates, imidophosphates and thiophosphates, can be generated.[60-62] A serious limitation of this methodology has been the instability of the reactive intermediates (nucleoside phosphomonochloridites or monotetrazolides) towards hydrolysis and air oxidation. This problem has been circumvented by either preparing the reactive species immediately prior to use or storing the active phosphite as a precipitate in hexanes at -20°C. This

problem has recently been solved by the development of a new class of nucleoside phosphites - the nucleoside N, N-dimethylaminophosphoramidites (Fig. 7).[63] These compounds are easily prepared by standard organochemical procedures, are stable under normal laboratory conditions to hydrolysis and air oxidation, and are stored as dry, stable powders. Activation

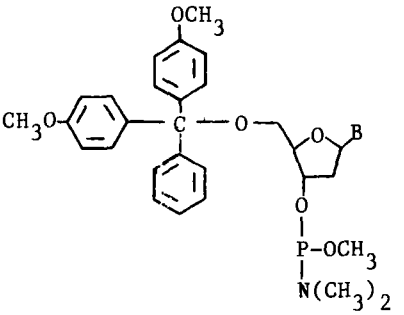

Figure 7.

by mild acid treatment (amine hydrochlorides, 3-nitrotriazole, or tetrazole) in the presence of a second, appropriately protected nucleoside (II: R_5, hydrogen; R_2, levulinyl) occurs very rapidly and the dinucleotide forms in essentially quantitative yield within 10-15 minutes.

The phosphite coupling approach has been used for synthesizing deoxyoligonucleotides[51,55,63] and oligoribonucleotides[59] on silica gel polymer supports. The synthesis begins by condensing a nucleoside (I: R_1, di-*p*-anisylphenylmethyl; R_4, hydrogen) with silica gel containing a carboxylic acid.[51] The resulting nucleoside (I: R_1, di-*p*-anisylphenylmethyl; R_4, silica gel) is treated with $ZnBr_2$ to remove the trityl ether[64] and then condensed with nucleoside N, N-dimethylaminophosphoramidites (a general structure is shown in Fig. 7). Thus, syntheses using this approach start at the 3'-end of a deoxyoligonucleotide and proceed in a 5'-direction. Yields of biochemically active deoxyoligonucleotides are essentially quantitative.

The scope of this review has been limited primarily to deoxyoligonucleotide synthesis. However completely parallel experiments with somewhat different chemical consequences have also been developed in the oligoribonucleotide series.[16,17]

Conclusions - Recent advances in the sequencing and synthesis of DNA have been enormous. Methods are presently available for the routine sequencing of any known DNA irrespective of size. Rapid, versatile synthetic methodologies are available for the synthesis of deoxyoligonucleotides. The combined impact of these developments on our understanding of biological regulation and differentiation should be enormous. We are now in a position to routinely probe the functional significance of gene control regions at the molecular level and to synthesize genes coding for medicinally useful proteins.

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Chapter 28. Recent Developments in Peptide Synthesis

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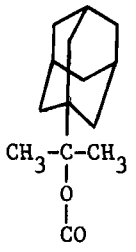
Introduction - Development in the synthesis of peptides has reached a plateau on which more work is devoted to the improvement of known basic principles and their adaption to problems of increasing difficulty than to the development of really new methods. This chapter deals primarily with progress reported in 1980, but also refers to papers published in 1979 and some in 1978, which are considered to be important for the present state of the art.

Techniques of peptide synthesis have been reviewed previously in this series in volume 7 (1972), and are extensively described in a handbook covering the literature through 1975.[1] A series covering the literature through 1977 continues to be a rich source of current information.[2] Proceedings of the American,[3-5] European[6-9] and Japanese[10-13] peptide symposia have been published, the latter being printed in English since 1977. Excellent information about all domains of peptide chemistry is provided by a new series.[14-16] Partial syntheses are described in two symposium proceedings.[17,18] A collective volume surveys relevant aspects of chemical, partial, and bacterial synthesis of peptides and proteins; it includes physical and biological aspects and provides an indication of future trends.[19]

Protecting Groups - These have been treated in a special volume[16] and an article.[20] Some new amino protecting groups have been examined.[21] Most syntheses are carried out with acid-labile protecting groups, e.g. benzyl-type residues for functional groups in the side-chains and the t-butyloxycarbonyl group in the N^{α}-position.[22] With t-butyl type residues for side-chain protection, t-aralkyloxycarbonyl groups have to be used for N^{α}-protection, because they are more acid-sensitive. Adpoc (1) is a recent addition to this family.[23] Alternatively, side-chain protection by t-butyl type residues and benzyloxycarbonyl or Fmos (2) groups for N^{α}-protection require only mild deblocking conditions. Fmoc is cleaved under β-elimination by weak bases, preferably piperidine.[24]



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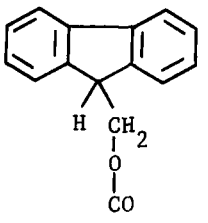

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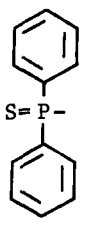

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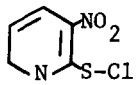

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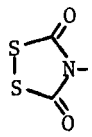

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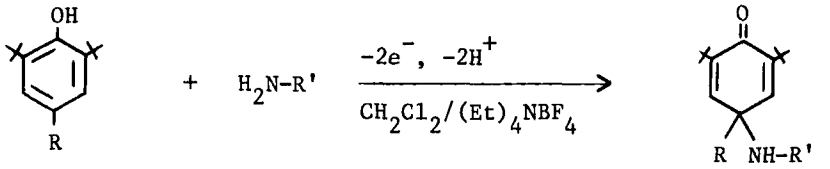
2-Methylthioethyloxycarbonyl is β-eliminated after alkylation and oxidation to the sulfoxide;[24] additionally it is cleaved by BrCN.[25] Diphenylthiophosphinoyl (3), which is analagous to diphenylphosphinoyl,[24] is introduced via the chloride which reacts with amino, hydroxyl and mercapto groups.[26] It is removed by dilute acid. 3-Nitro-2-pyridinesulfenyl (Npys) chloride (4) also reacts with both amino and hydroxy groups.[27] The Npys group is stable to trifluoroacetic acid and 88 % formic acid, but is easily removed by 0.1 - 0.2N HCl in dioxane. Kinetics and mechanism of the thiolytic removal of the dithiasuccinoyl (Dts) amino protecting group (5) have been studied.[28] Hydrolyzable protecting groups are easily cleaved by transfer hydrogenation using ammonium formate and Pd/C in methanol or dimethylformamide.[29] Further deblocking agents for protolytically cleavable groups are thioanisole-trifluoroacetic acid,[30] thioanisole-methanesulfonic acid[31] and thioanisole-trifluoromethanesulfonic acid.[31] Dimethylsulfide-methanesulfonic acid will also remove the methoxycarbonyl (Moc) protecting group.[32]

Sterically hindered phenols of type (6) are electrochemically oxidized providing a cyclohexadiene N-protecting group (PChd) which is stable to alkali, but may be removed by 50 % trifluoroacetic acid in CH_2Cl_2.[33]



6 PChd

N^{im} protection of histidine is important with regard to enhanced racemization of histidine with a free imidazole moiety. The phenacyl[34] and the 4-methoxyphenylsulfonyl group[35] have been recommended. They are removed by nucleophilic attack.

The N^{in}-formyl group on tryptophan helps to prevent t-butylation during the cleavage of t-butyl groups or Boc residues with trifluoroacetic acid or HF.[36]

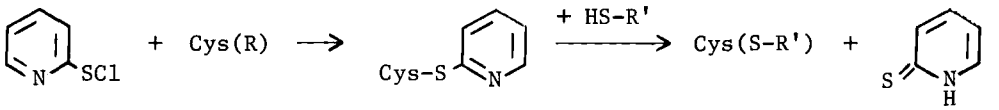
The protection of the guanidino group in arginine has been approached in several ways. The mesitylene-2-sulfonyl[37] and the 4-methoxy-2,6-dimethylbenzenesulfonyl (Mds) groups[38] have been studied, both being cleaved by HF, methanesulfonic acid and trifluoromethanesulfonic acid/trifluoroacetic acid (1 : 1). N^G-Tosyl-arginine is deprotected by thioanisole/trifluoromethane-sulfonic acid.[39] The nitro group is selectively removed from arginine by titanium (III).[40] Two Boc residues have been introduced into the guanidino group, but the yield was low.[41]

The present state of the art in the protection of hydroxyl groups of serine, threonine, tyrosine and other hydroxyl containing amino acids,[42] as well as protection of carboxyl groups,[43] has been described. The previously unknown tryptophan ethyl ester tosylate and additional amino acid ethyl ester tosylates are prepared with ethyl tosylate in refluxing ethanol.[44] The cyclohexyl ester, as a protecting group for aspartyl peptides, minimizes aspartimide formation during acidic and basic treatments.[45]

Simple peptides have been synthesized using $[(NH_3)_5Co(III)]$ as a carboxyl protecting group,[46] providing a red color and favorable solu-

bility properties to the protected peptides. Deblocking is achieved with sodium sulfide. 2-Haloesters and triethylamine form choline esters, which are both cleaved at pH 10 - 11.[47] The 4-sulfobenzyl residue as an acid-stable carboxyl protecting group increases the solubility of peptides and is split off by alkali and catalytic hydrogenation.[48] Thioanisole/trifluoromethanesulfonic acid cleaves the methyl ether in O-methyl-tyrosine.[49]

Protection and deblocking of the mercapto group of cysteine have been treated extensively.[50] The solubilizing S-(2-sulfobenzyl)cysteine or S-[2-(sulfomethyl)benzyl]cysteine derivatives are split by Na/NH_3 or tributylphosphine;[51] 4-pyridylmethyl is cleaved by electrolysis at pH 2.6.[52] The photocleavable 2-nitrobenzyl residue has now been applied to cysteine.[53] As a variation of the known and useful S-(methoxycarbonylsulfenyl)cysteine,[54] the corresponding t-butyl- and benzyloxycarbonyl analogues have been prepared.[55] The stability of methionine and histidine with a protonated N^{im} during treatment of S-trityl- and S-(acetamidomethyl)cysteine with iodine was assessed.[56] S-(2-nitro-1-phenylethyl)cysteine is formed by addition of cysteine to 2-nitrostyrene. The protected derivative is stable under conditions normally used for acidic cleavage of N- and O-protecting groups and can be removed under mildly basic conditions insufficient to cleave ester bonds.[57] 2-Pyridinesulfenyl chloride (7) attacks S-protected cysteine, forming a reactive intermediate which on treatment with HS-R′ yields asymmetric disulfides.[58] Application of the most common procedures to the formation of asymmetrical cysteine peptides has been reviewed.[54]



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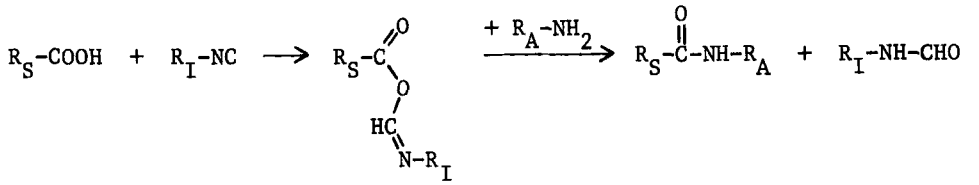
R = trityl (Trt), diphenylmethyl (Dpm), acetamidomethyl (Acm), t-butyl (Bu^t), t-butylmercapto (SBu^t).

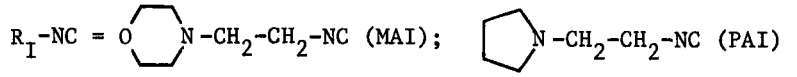
Alkylation and oxidation of the sulfur in methionine are reversible reactions and can be used to protect the sulfur temporarily. Several papers have dealt with the utilization and reduction of Met (O) e.g. by N-(methylmercapto)acetamide and other thiols[59] or iodide.[60] The use of S-(t-butylsulfenium)methionine has been demonstrated in the synthesis of substance P.[61] It is also recommended as a protection for methionine during iodination. The sulfenium structure increases the solubility of methionine containing peptides and facilitates their purification. The t-butyl bond is cleaved by heating an aqueous solution at 50° C for 3 hours.

Methods of Peptide Bond Formation - This subject has been extensively reviewed;[14,15,19] further information can be found in the proceedings of recent peptide symposia.[5,19,13] A reassessment of the N-carboxyanhydride (NCA) method has appeared,[62] dealing with optimization of conditions and minimization of by-product formation. The NCA method is also useful for the preparation of peptides containing α, α-disubstituted amino acids.[63] A review of the rearrangement of acid adducts of push-pull acetylenes and its application to peptide synthesis has appeared.[64] Cyclic analogues of eledoisin were synthesized[65] using the 4-component condensation;[66] 9-formylfluorene can be used as the aldehyde in this reaction.[67]

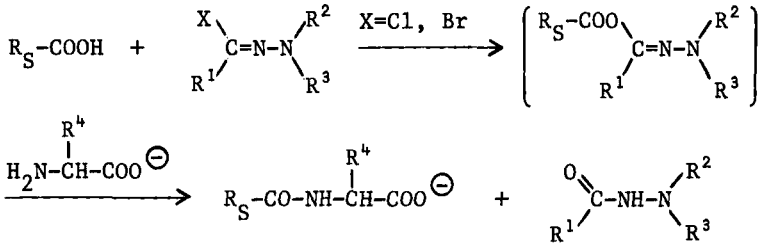
Several novel reagents have been described. Isonitriles are useful

condensing reagents; in the case of steric hindrance they seem to be superior to carbodiimides.[68]



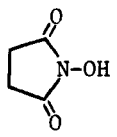


Imidoyl halides react with carboxylic acids to form an activated derivative; N-protected peptides are obtained on reaction of this intermediate with free amino acids.[69]

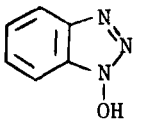


Active esters are still the most frequently used compounds in peptide synthesis, often without isolation in the carbodiimide procedure. HOBt (9) is most widely applied, but (8) is also suitable in most cases. HOBt as a catalyst in the aminolysis of activated esters is used in a large-scale synthesis of somatostatin.[70] HOOBt (10) seems to be the reagent of choice in minimizing racemization.[71] The pentachlorophenyl ester of an N-protected peptide has been reacted with a peptide ester at 100° - 105°C in high vacuum without racemization.[72]

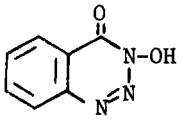
Recently, 4-oximinopyrazol-5-ones (11) have been proposed as novel activating hydroxyl components;[73] no racemization was noted in the Weygand test. Two new reagents, N,N'-disuccinimidyl carbonate (12)[74] and N-(succinimidyl)diphenyl phosphate (13)[75] have been applied to the preparation of N-hydroxysuccinimide esters. Premature cleavage of the Fmoc group (2) in the application of Fmoc-amino acid active esters can be kept to a minimum.[76]



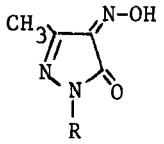
HONSu (8)



HOBt (9)



HOOBt (10)



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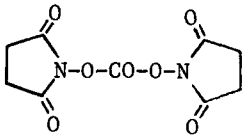

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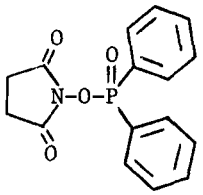

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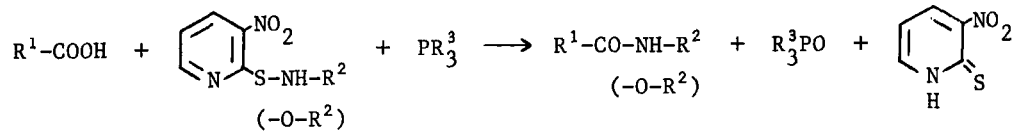
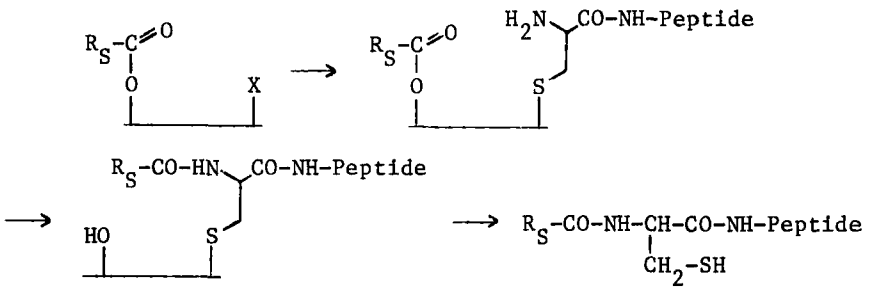
Several papers dealing with organophosphorous reagents have appeared. Bradykinin was synthesized with tertiaryphosphane/hexachloroethane;[77] racemization is minimized in this reaction with HOBt as an additive. Another coupling reagent is triphenylphosphine/SO_3.[78] A study was carried out with n-propylphosphonic acid anhydride.[79] Diphenylphosphinic mixed anhydrides are useful in the acylation of sterically hindered amino groups and in solid-phase synthesis.[80] N-Npys and O-Npys groups are activated by tertiary phosphine, forming a peptide or ester linkage with N-protected amino acids or peptides.[81]



N-terminal cysteine capture has been proposed to yield cysteine peptides in the following way:[82]

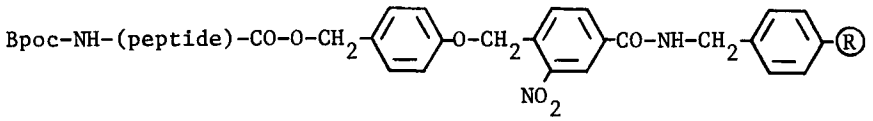


Racemization - Although great effort has been made to develop racemization-free syntheses,[83] there is no method yet available which completely avoids this complication. Best results in critical cases have been obtained with the mixed carbonic anhydride method,[84] with azide coupling[85] and DCC/HOObt.[71] Unfortunately, only poor yields are often obtained in the condensation of larger segments via azides. Results with organophosphorous reagents, e.g. n-propylphosphonic anhydride (PPA), are promising.[71,79] In the N-terminal elongation of peptides with an unprotected C-terminal carboxyl group, activation using HOBt should be avoided, since it always causes extensive racemization.[86,87]

Histidine shows an enhanced tendency to racemize, especially as long as the imidazole nitrogen is free. N^{π}-protected histidine yields optically pure peptides with suitable coupling methods, whereas N^{τ}-isomers suffer gross racemization.[88] In the dipeptide pGlu-His-OH, the unprotected histidine racemized to an extremely high extent (23 % with DCC/HOBt; 15 % with DCC/HONSu). DCC/HOBt surprisingly did not diminish the racemization rate of N^{im}-(dinitrophenyl)histidine in pGlu-His(Dnp)-OH. With DCC/HONSu or PPA the degree of racemization was 6 %, whereas with DCC/HOObt the resulting peptide was optically pure.[71] Only the stepwise synthesis with N-urethane-protected amino acids proceeds usually racemization-free.[89,90] 2-Alkoxyoxazolones, if formed, are much more chirally stable than 2-alkyloxazolones.[91]

Other Side Reactions - The occurrence of side reactions during peptide bond formation and deblocking procedures is still a major problem in the preparation of larger peptides.[92] Aspartimide formation not only occurs in the synthetic steps, but also during purification and storage of aspartyl peptides, most frequently at the Asp-Gly bond.[92,93] During acidic deprotection of peptides, side reactions are mainly caused by the attack of cations on Met, Cys, Tyr and Trp and by N to O acyl shift and cleavage of the peptide chain. Scavengers satisfactorily minimize alkylation.[92]

The Polymer-Supported Synthesis - This method of peptide synthesis further demonstrated its usefulness, branching off into solid phase[94] and liquid phase[95] techniques. Inherent problems such as the inevitable appearance of failure sequences are now under better control. The adoption of segment coupling extends the scope of these methods further. Two authoritative reviews[94,95] and a lucid treatise on the role of the solid in solid-phase synthesis have appeared.[96]



14

Photolabile, multi-detachable p-alkoxybenzylalcohol resin supports have been synthesized. Treatment of Bpoc-protected peptide (14) with 50 % trifluoroacetic acid provided the free peptide, whereas photolysis gave Bpoc-peptide-p-hydroxybenzylester, which after alkaline 1,6-elimination, gave the Bpoc-protected peptide.[97] This compound can be used again in segment condensation. In either dichloromethane or dimethylformamide, the space available for peptide chain growth within the swollen resin beads is not a limiting factor in solid phase peptide synthesis. Due to the influence of the growing peptide chain on swelling, the space potentially available actually increases rather than being gradually filled by peptide.[98] The use of a polyamide-based, solid phase method permitted the rapid and efficient synthesis of gastrin peptide amides.[99] In the synthesis of a cytochrome C (66-104)-segment by a polymer-supported method, the N^{ε}-group of lysine was protected by trifluoroacetyl; Boc as N^{ε}-protecting group led to internal aggregation and lowered the yield.[100]

Enzymatic Peptide Synthesis - Forty years after a first successful attempt to reverse the enzymic cleavage of peptide bonds, this approach to peptide synthesis has been revived.[101] Carboxypeptidase Y catalyzes the condensation of N-protected amino acid or peptide esters with amino acid or peptide amides.[102] Deamidation is catalyzed by the same enzyme under different conditions.[103] α-Chymotrypsin promotes coupling in aqueous organic solvent systems.[104] Papain, trypsin[105] and thermolysin[106] are also used. Condensation of ribonuclease S-peptide segments (1 - 10) and (11 - 15) was achieved with clostripain using S-protein (21 - 124) as a kinetic trap.[107] Human insulin was formed by trypsin-catalyzed condensation of des-Ala^{B30}-porcine insulin with Thr-OBu^{t} and subsequent treatment with trifluoroacetic acid.[108]

Semisynthesis - Total synthesis of proteins and many larger peptides is either impossible or uneconomical. However, specific reactions can be carried out, e.g. with insulin[17,18,109] and, normally after selective enzymatic cleavage, with many proteins.[17] A disadvantage of these chemical procedures is the necessity to work with minimal protection.[17,18] Several articles provide a good overview of the scope and limitations of characteristic reactions in this relatively new field.[110-112] B30-Alanine of porcine insulin is split off by carboxypeptidase A[113] and subsequently substituted by threonine,[108] thus providing human insulin by simple semisynthesis.

Purification and Purity of Synthetic Peptides - This is still a crucial problem and deserves great attention. The increasing efficiency of analytical methods is an exacting challenge to improve purification techniques. Preparative HPLC has emerged as a superior method and is treated in numerous papers and an excellent survey on the state of the art.[114]

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Chapter 29. Drug Metabolism

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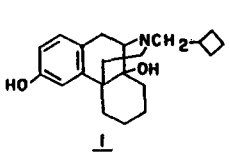
Introduction - In this chapter, we present a selective compilation of contributions from the drug metabolism literature of the past two years, which may assist the medicinal chemist in using biotransformation, pharmacokinetic, analytical and clinical data in the design of new drug entities. The literature of drug metabolism is rapidly proliferating, due to factors which include rapid advances in methodology, demands of drug regulatory agencies and unraveling of the mechanisms involved. Three new journals have appeared: Biopharmaceutics and Drug Disposition, Therapeutic Drug Monitoring and the European Journal of Drug Metabolism and Pharmacokinetics.

The fourth volume of the series Fate of Drugs in the Organism: A Bibliographic Survey,[1] the third volume of Drug Fate and Metabolism,[2] the fourth volume of Progress in Drug Metabolism[3] and the fifth volume of Foreign Compound Metabolism in Mammals[4] have been published. Two recent books of general interest deal with human[5] and extrahepatic drug metabolism.[6]

Methods - The use of immunoassays as an analytical method for drugs has expanded in the last few years. The more common tracers such as ^{125}I have been used in a radioimmunoassay for cyclazocine,[7] and ^{3}H has been used for the radioimmunoassay of butorphanol (1),[8] bruceantin (2)[9] and flurazepam (3).[10]



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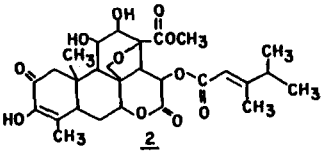

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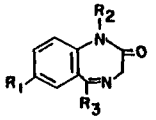
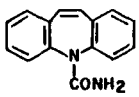
Enzyme immunoassays for lidocaine[11] and for carbamazepine (4)[12] have demonstrated the utility of nonisotopic tracers in immunoassays. The use of an "antibody electrode" demonstrates the flexibility of the enzyme immunoassay.[13] A fluorescent label has been used in an immunoassay for phenytoin.[14]

Numerous detectors continue to be used in gas liquid chromatography (GLC) and high performance, or pressure, liquid chromatography (HPLC). Electron capture and nitrogen-selective detectors have been used for the determination of nitrazepam (5) by GLC.[15] Nitrazepam and other benzodiazopines have also been analyzed by HPLC using amperometric detectors.[16] The use of electrochemical detectors has been recently reviewed.[17] Electrochemical detectors have also been used for the determination of morphine[18] and for 5-methyltetrahydrofolic acid.[19]



3 R_1 = Cl, R_2 = $CH_2CH_2N(C_2H_5)_2$, R_3 = C_6H_4-o-F
5 R_1 = NO_2, R_2 = H, R_3 = C_6H_5



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Kaye[20] has reviewed the GLC determination of over 300 drugs. The practical aspects of HPLC as an analytical technique have also been reviewed.[21] Ahuja[22] has reviewed the chemical derivatization for HPLC. Theory, column selection, derivatization, detectors and analysis of various classes of drugs using GLC and HPLC have been reviewed.[23] The use of gas chromatograph-mass spectrometry (GC-MS) is increasing. Jenden[24] has reviewed the use of selected ion monitoring and the trend of GLC, MS, computer and interfaces operating together as a functional unit. Techniques for GC-MS have also been reviewed by Budde.[25] The use of LC rather than GC in combination with MS has been reviewed[26] and a specific example of its utility shown in the LC-MS analysis of nonvolatile samples.[27]

Colorimetric assays continue to have a place in analysis of drugs, such as procaine hydrochloride[28] and chlorhexidine.[29] Fluorometry has been used to determine butaperazine in plasma,[30] and a new laser-induced fluorometric method has been developed to analyze intrinsically fluorescent compounds.[31]

Pharmacokinetics - Review articles have appeared on the clinical pharmacokinetic aspects of diuretics,[32] antiarrhythmic agents,[33] drug use in childbirth[34] and in the postoperative period,[35] and on extracorporeal hemoperfusion techniques.[36] Drug bioavailability has been reviewed;[37] the effects of smoking,[38,39] age,[39] food intake[40] and disease[41] on these studies using model compounds was reported. Blood sampling through cannulas flushed with heparinized saline altered plasma binding,[42] in comparison with samples obtained by venipuncture.

The pharmacokinetics of benzodiazepines have been intensively studied with regard to the effects of subjects' age and sex. The elderly had a mean eleven hour prolongation of the half-life of nitrazepam,[43] a mean prolongation of forty-eight hours for diazepam [44] and no prolongation with lorazepam.[45,46] Using the half-life of the drugs as a measure of metabolizing ability may be misleading, as age may alter the volume of distribution; age increased both the half-life and volume of distribution for diazepam[46] and reduced clearance.[46,47] Diazepam plasma clearance is less in women than in men,[48] but age had little effect.[47] Differences in the kinetics of the short-acting and long-acting benzodiazepines were reviewed with emphasis on the half-lives of the metabolites formed.[49] Diurnal variations in total plasma diazepam, unbound diazepam and N-desmethyldiazepam were reported; total diazepam concentrations were lowest at night and highest during the day, while both free diazepam and N-desmethyldiazepam were highest before 8:00 a.m. and significantly lower during the daytime.[50] Disulfiram prolonged the half-life and reduced the total body clearance of chlordiazepoxide and diazepam in both normal and alcoholic patients; disulfiram had no effect on the kinetics of oxazepam but reduced the half-life and clearance of lorazepam.[51] Plasma concentrations of lorazepam were fitted to a two-compartment body model.[52] The placental transfer of chlorazepate was slower than that of its metabolite, nordiazepam.[53] The biotranformation of clorazepate into nordiazepam was significantly higher in pregnant women, compared to non-pregant women.[54] The transfer of diazepam and nordiazepam across the human placenta in vitro was greater than the transfer of clorazepate.[55] Highly neurotic subjects absorbed diazepam more rapidly than less neurotic subjects, probably due to shortened gastric emptying time.[56]

An open two-compartment body model described the plasma concentration profile of orally administered zomepirac, a nonnarcotic analgesic; in healthy male volunteers the terminal half-life was 4.3 hr.[57] Cimetidine, an antisecretory agent, had a terminal half-life of 1.6 hr[58] and did not affect the bioavailability of concurrently administered enteric-coated prednisolone.[59]

Meperidine and normeperidine levels in fetus and mother indicated fetal/maternal ratios of 0.86 and 0.87, respectively;[60] the appearance of normeperidine in the mothers' plasma slowly increased for ten minutes, increased rapidly for twenty minutes and then slowly thereafter.[61] Babies, born at term, received naloxone via the umbilical vein; mean plasma half-life, apparent volume of distribution and plasma clearance were 2.65 hr, 1.78 l/kg and 576 ml/kg/hr, respectively.[62]

Simultaneous oral doses of unlabeled and isotope-labeled maprotiline, an antidepressant,[63] and benoxaprofen, an antiinflammatory,[64] were used for estimates of bioavailability and drug kinetics; the use of stable isotopes has recently been reviewed.[65] Differing gastrointestinal side effects of indomethacin and sulindac result from exposure of the intestinal mucosa to active drug in the former case and to inactive prodrug with the latter.[66] A model of enterohepatic circulation in the rat has demonstrated that long apparent half-lives may be artifacts of recirculation.[67]

Several novel methods for determining pharmacokinetic parameters have been reported. By using only the area under the plasma concentration versus time curve, the maximum plasma concentration and the time to the maximum concentration, one can determine the equivalence of the apparent first-order rate constants for absorption and elimination in a one-compartment body model.[68] Apparent first-order absorption rate constants for a one-compartment body model can be determined from blood or urine data without using nonlinear regression analysis.[69] A model-independent polyexponential approach to bioavailability was developed using neither absorption, distribution or elimination rate constants nor a volume of distribution term.[70]

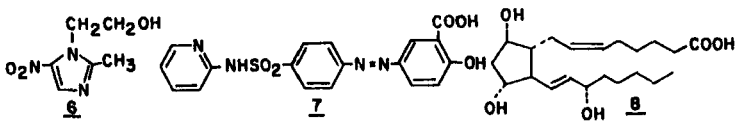
Novel Biotransformation Pathways - The recent literature contains three examples of novel conjugation pathways. The first involves the addition of CO_2 to the amino nitrogen of tocainide, an experimental antiarrhythmic, followed by glucuronic acid conjugation.[71] In the second, the formation of a carbon-glucuronide bond has been demonstrated in the C-glucuronidation of the acetylenic moiety of ethchlorvynol in the rabbit.[72] In the third, some 12% of the urinary metabolites of phenol were conjugated with phosphate in sheep; this may be a function of the rumen since this was not found in the rat or pig.[73]

The rupture of the C-H bond has been found necessary for the defluorination of methoxyflurane, although it is not rate limiting.[74] Noscapine was metabolized via C-C bond cleavage to produce meconine, cotarnine and hydrocotarnine.[75] The N-formylation of an aromatic amine, 2-aminoanthraquinone, may account for the nephrotoxicity of this compound.[76] The metabolic formation of glycerol esters of 3-phenoxybenzoic acid resulted in the retention of the 2'- and 3'-(3-phenoxybenzoyl)dipalmitins in the skin of rats.[77] A hydroxy metabolite of DDT[78] and hydroxycannabinols[79] are conjugated with fatty acids to form neutral, persistant metabolites.

Drug Disposition - The roles of both non-enzymatic reactions[80] and the specific enzyme systems involved in drug metabolism (mixed function oxidases,[81,82] epoxide hydratase[83,84] and carbonyl reductases[85]) have been reviewed. The significance of detoxication reactions in clinical practice[86,87] and toxicology,[88] including the effects of biotransformation on toxicity of inhalation anesthetics,[89] was reported. A summary of the influence of intestinal microflora on drug metabolism was published.[90]

Metronidazole (6)[91] and sulfasalazine (7)[92] were reduced by bacterial enzymes; the latter compound is converted to 5-aminosalicylic acid, which undergoes capacity limited N-acetylation during passage through the intestinal

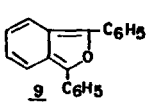
wall. Pentobarbital[93] was only one-third bioavailable after oral administration; intestinal metabolism accounted for 42% of the reduction, with hepatic metabolism being responsible for the remainder. The oxidation of oxamniquine to a 6-carboxylic acid during absorption from dog intestine was reported; the conversion of the 6-hydroxymethyl group to the carboxylic acid was not inhibited by either gentamicin or erythromicin, and the same product was found in a rat everted intestinal sac.[94] Prostaglandin $F_{2\alpha}$ (8) was metabolized by an isolated segment of rat jejunum to 15-keto- and 13,14-dihydro-15-keto- derivatives; infusion of an ester yielded, in addition, the free acid and the 13,14-dihydro-acid.[95] Disease states, such as diabetes, altered the metabolism of 7-ethoxycoumarin[96] and benzo(a)pyrene[97] by rat gut and lung microsomes.



Cultures of human lung conjugated benzo(a)pyrene and 1-napthol with sulfuric acid; in contrast, rat lung cultures conjugated with glucuronic acid.[98] Tissue from human pulmonary tumors oxidized benzo(a)pyrene to dihydrodiols, with the 7,8-dihydrodiol representing 40% of the total dihydrodiol content.[99] Differences in activities of coumarin hydroxylase and 7-ethoxycoumarin deethylase among viable cells isolated from rabbit lung suggest that at least two cell types are responsible for these activities.[100] 3-Methylcholanthrene, which induces drug metabolizing enzymes in regenerating rat liver, failed to stimulate metabolism of model drug substrates by rabbit lung during the period of compensatory growth following unilateral pneumonectomy of rabbits; it was suggested that different regulatory mechanisms are involved in liver and lung.[101] Uptake of imipramine[102] by rat and rabbit lung removed about 80% from the perfusing medium; the N-oxide was the major metabolite, with small quantities of desipramine and hydroxylated metabolites identified in the rat preparation. Rabbit lung only transformed about 3%, while rat lung converted about 76% of the imipramine. The isolated perfused rabbit lung accumulated morphine[103] and chlorpromazine[104] by passive diffusion. Morphine and ethylmorphine[103] were not metabolized, but chlorpromazine was dealkylated by rabbit lung and converted to the N-oxide by rat lung. SKF 525a inhibited chlorpromazine metabolism in rabbit lung but stimulated N-oxidation in the rat.[105] In contrast, chlorphenteramine was actively accumulated by isolated rabbit lung[106] and lung slices.[107] Estrogen dependence of the inducible enzymes aryl hydrocarbon hydroxylase and 7-ethoxycoumarin-O-deethylase was demonstrated.[108] Methotrexate resistance of murine Lewis lung tumor was related to both low membrane permeability and high levels of dihydrofolate reductase.[109]

Drugs with high lipophilicity are distributed to the pancreas, even during secretin or pancreozymin infusion;[110] the ratio of the concentration of dimethadione in pancreatic juice to plasma was increased by secretin, while pancreozymin had no effect on dimethadione, isonicotinamide, isonicotinic acid, sulfanilamide, sulfanilic acid, sulfisoxazole or procainamide ratios. Uptake of benzo(a)pyrene by the pancreas was only 16% that of the liver, leading to a thousand-fold difference in the amount excreted in pancreatic juice compared to bile.[111] Methylcholanthrene pretreatment increased only biliary excretion of benzo(a)pyrene, while either secretin or pancreozymin increased pancreatic clearance, exclusively. Murine pancreatic islet cells accumulated chloroquine;[112] other weak organic bases, such as methadone or propranolol, accumulated in the mitochondria, and decamethonium, a strong base, was associated with the granule-rich fraction of pancreatic homogenates.[113]

The concentration-dependent interconversion of salicylic and salicyluric acids by isolated rat kidney was unexpected.[114] This organ was shown to catalyze the 3-O-methylation of isoproterenol, with the metabolite appearing in urine and accumulating in kidney tissue.[115] Isolated rat kidney cells could conjugate acetaminophen with glucuronic acid, sulfuric acid, cysteine and N-acetylcysteine.[116] Microsomes from the medulla of rabbit kidney carry out a prostaglandin cyclooxygenase-mediated, arachidonic acid-dependent conversion of 1,3-diphenylisobenzofuran (9) to o-dibenzoylbenzene, while microsomes from the cortex require an NADPH-dependent mixed function oxidase.[117]



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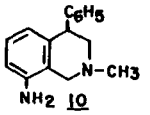

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A number of reviews of maternal, fetal and neonatal metabolism has appeared.[118-121] Pregnancy did not significantly affect the disposition of nomifensine (10) in rats,[122] phencyclidine in pigs,[123] primidone[124] and meperidine[125] in humans or salicylate binding in sheep.[126] Accumulation of carbaryl by rat fetal brain, heart and lung[127] and of morphine by monkey and human lung and meconium[128] was reported. In humans, pregnancy decreased plasma phenytoin levels without affecting the urinary excretion pattern of the metabolites;[129] in rats, pregnancy impaired conjugation with glucuronic acid and elevated plasma concentrations.[130] Clearances of phenytoin, carbamazine and phenobarbital fluctuated during pregnancy and peaked near term.[131] Metabolism of benzodiazepines by fetal liver fragments and isolated hepatocytes[132] and the effects of fetal development on steroid-glucuronyl transferase,[133] plasma cholinesterases[134] and mixed function oxidase[135] activity were reported.

Pretreatment with liposomes alters the tissue distribution of liposome encapsulated inulin in mice.[136] Differences in disposition and serum half-life were observed in the rabbit with gentamicin incorporated in either acid, basic or neutral liposomes.[137] Triamcinolone incorporation into liposomes reduced percutaneous absorption and increased its localization in the skin.[138] NMR studies with chlorpromazine suggested that incorporation of drug into the membrane takes place near the carbonyl groups of lecithin.[139]

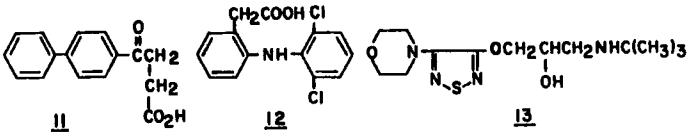
The metabolism of tetrahydrocannabinols (THC) has been explored in a number of species. Over 20 acidic metabolites of Δ^9-THC were isolated from rabbit urine.[140] Mouse liver contained 28 metabolites of Δ^8-THC,[141] and a lesser number of acidic metabolites were found in mouse, guinea pig and rabbit livers[142] after Δ^9-THC administration. A Δ^9-THC-11-oic acid glucuronide was identified in human urine.[143]

Fenbufen (11) was metabolized in animals[144] and man[145] by reduction of the keto group, hydroxylation of the α- or β-carbon in the butanoic acid moiety, 4'-hydroxylation of the biphenyl moiety and by β-oxidation through the same pathway by which fatty acids are metabolized. Species differences in metabolism were discussed.

Diclofenac (12), an anti-inflammatory agent, was extensively metabolized by animals and man.[146] In rat, baboon and man, metabolism involves conjugation of parent drug, or oxidation of the aromatic rings, followed by conjugation. The sites of hydroxylation were either the 3 or 4 position of the dichlorophenyl ring or position 5 of the phenyl ring attached to the acetic acid moiety. Dogs do not

appear to oxidize diclofenac and excrete primarily a taurine conjugate of intact drug.

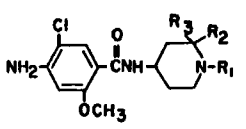
The metabolism of timolol (13) was compared in animals and man.[147] The oxidative opening of the morpholine ring and the oxidation of the oxypropanolamine side chain appear to be mutually exclusive pathways, the former being of greater importance in rodents and man, and the latter in the dog. In contrast, 4-ethoxy-2-methyl-5-morpholino-3-(2H)-pyridazinone was metabolized mainly by opening of the morpholine ring in mice, rats, rabbits, dogs and monkeys.[148]



11 12 13

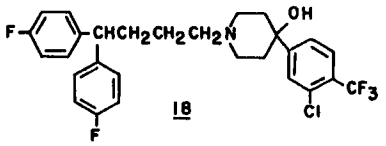
The disposition and pharmacokinetics of zomepirac, an analgesic agent, in animals and man were reported.[149] Intact zomepirac and its glucuronide were the major urinary metabolites in man and monkeys. Hydroxyzomepirac was the major component in hamster urine and was also present in significant amounts in the urine of rats and mice. Further oxidation to 4-chlorobenzoic acid occurred in both rat and mouse.

Clebopride (14) was extensively metabolized by rat, rabbit, dog and man, with up to 25 metabolites detected in urine.[150] The major metabolites were: N-(4'-piperidyl)-2-methoxy-4-amino-5-chlorobenzamide (15); N-(4'-piperidyl-2'-one)-2-methoxy-4-amino-5-chlorobenzamide (16); and N-(1'-α-hydroxybenzyl-4'-piperidyl)-2-methoxy-4-amino-5-chlorobenzamide (17). In addition, 2-methoxy-4-amino-5-chlorobenzoic acid was detected in the dog. Rabbit microsomes were also able to cleave the amide bond and N-debenzylate clebopride.[151]



14 R_1=CHC_6H_5, R_2=R_3=R_4=H
15 R_1=R_2=R_3=H
16 R_1=H, $R_{2,3}$==O
17 R_1=CHC_6H_5, R_2=R_3=H, R_4=OH

Quantitative metabolism and disposition of penfluridol (18), a neuroleptic agent, were studied in several species.[152] The major pathways of metabolism in the rat, rabbit, dog and man were oxidative dealkylation, followed by β-oxidation, conjugation of penfluridol and conjugation of the acidic metabolites. The qualitative and quantitative characterization of the 4-[4-chloro-3-(trifluoromethyl)phenyl]-4-piperidinol metabolite formed after N-dealkylation has also been reported.[153]



18

It was possible to separate and unequivocally identify p- and m-hydroxymephenytoin after isolation from dog urine.[154] Several glucuronides and new metabolites of mephenytoin were identified in human urine.[155] The urinary excretion of hydroxylated metabolites was stereoselective and dose-dependent, while the demethylation of mephenytoin was not truly stereoselective.[156] Differences in the enantiomeric hydroxylation of oxprenolol could reflect either

the enantiomeric composition of oxprenolol reaching metabolic sites in the liver or the substrate stereoselectivity in the metabolic process.[157]

Some commonly held tenets of drug metabolism have recently been shaken. Weitering et al.[158] have concluded that the relative decrease of phenol sulphation at high phenol doses is not due to sulphate depletion but may be explained by differences in the affinity of phenol for UDP-gluconyltransferase and phenolsulphotransferase. Microsomal studies have indicated that N-hydroxy-acetaminophen is not the reactive metabolite of acetaminophen responsible for macromolecular binding.[159] The majority of the irreversible binding of chlor-amphenicol to protein in vivo is not via a reactive metabolite as is seen in vitro but instead is due to its metabolism into small fragments and subsequent biosynthesis into glycine and serine.[160]

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