ANNUAL REPORTS IN MEDICINAL CHEMISTRY Volume 14

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

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

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PREFACE

Since its inception, the objective of Annual Reports in Medicinal Chemistry has been to provide brief, critical summaries of highlights of the recent literature in the fields of chemistry, pharmacology, biochemistry and medicine for those interested in the discovery and development of new drugs. The format of organization originally established for this series has admirably served the intended purpose. Accordingly, as in the past, this year's volume is divided into six sections: CNS Agents; Pharmacodynamic Agents; Chemotherapeutic Agents; Metabolic Diseases and Endocrine Function; Topics in Biology; and Topics in Chemistry and Drug Design.

In selecting suitable chapter topics, the editors have sought a balance between areas of long-standing interest in drug research and special topics that may offer opportunities for future drug design and exploration. Similar thinking has guided the selection of subject matter for inclusion in many of the chapters. Thus, the discussions increasingly reflect the view that new breakthroughs in drug discovery are likely to emerge from new conceptual approaches based on a better understanding of disease processes and drug mechanisms.

This volume, for the first time, contains a cumulative chapter title index as a convenient reference for quickly locating reviews on special topics that have appeared in past volumes.

To the many individuals who contributed to this volume, we extend our sincere thanks.

Groton, Connecticut
May 1979

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Section I - CNS Agents

Editor: Leslie G. Humber, Ayerst Laboratories, Montreal, Canada

Chapter 1. Antidepressants

Roger M. Pinder, Organon International, Oss, The Netherlands

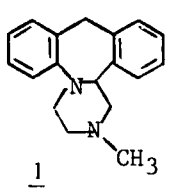
Introduction - Concern over the safety of tricyclic antidepressants (TCA)[1,2] has maintained the impetus to develop new drugs. Compounds reviewed in 1978 include mianserin,[3,4] nomifensin,[5] and all non-TCA.[6] Symposia covered dothiepin,[7] mianserin,[8] nomifensin,[9] and trazodone.[10] The uses of L-tryptophan and 5-hydroxytryptophan (5-HTP) in depression[11] and the properties of the new antidepressants[12-16] were summarized. The neuropharmacology of depression,[17] and the mechanism of action[18] and pharmacokinetics[19] of antidepressants were reviewed, and the monoamine theory reassessed.[20]

Previous reports[21] that some TCA and non-TCA, like iprindol and mianserin, were inhibitors of histamine-sensitive adenylate cyclase from mammalian brain in vitro were confirmed for a wide range of antidepressants.[22] The effect appeared to be mediated by H_2-receptors but neither monoamine oxidase inhibitors (MAOI) nor selective serotonin (5-HT) uptake inhibitors were very effective. However, TCA also blocked histamine-stimulated cyclic GMP formation in cultured nerve cells by a mechanism which involved H_1-receptors.[23] The relevance of these findings, and indeed of the role of histamine in the central nervous sytem, has still to be defined.

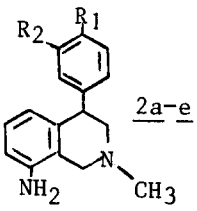
Evidence accumulated in 1978 for a catecholamine receptor supersensitivity theory of depression.[18] The therapeutic action of antidepressants may be due to delayed post-synaptic changes in receptor sensitivity, rather than to acute events like uptake. Various drugs, including TCA, mianserin, viloxazine and iprindol, as well as electroconvulsive therapy (ECT), but not selective 5-HT uptake inhibitors, caused central alpha-adrenoceptor subsensitivity in rats as measured by noradrenaline (NA)-associated adenylate cyclase or by receptor binding.[18] In vivo, the effects were associated with chronic but not acute treatment, paralleling the clinical effects. MAOI may cause similar effects on chronic but not acute treatment.[18,24-27] Brain NA turnover in rats was decreased by chronic desipramine and other TCA, but unaffected by iprindol and increased by mianserin.[3,28]

Safer Drugs - Mianserin (1) and nomifensin (2a, R_1=R_2=H) are effective antidepressants, superior to placebo and comparable to TCA. There have been no trials so far comparing 1 with 2a, but neither acts any faster than TCA. Both lack cardiotoxicity and have minimal anticholinergic effects, and while experience with nomifensin is limited, mianserin appears to be safe in overdosage.[29] Mianserin also has anxiolytic activity comparable to diazepam,[3] and can be given as a single bedtime dose or in divided doses. Nomifensin cannot be given at night and its short half-life necessitates divided daily dosage. Toleration of 1 is good in the elderly, and in a double-blind trial in geriatric depressives, 1 was superior in efficacy and

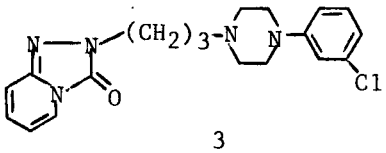
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1



2a-e



3

side-effects to amitriptyline.[30] Experimental studies in isolated hearts, intact animals, healthy subjects, depressed patients and patients with cardiac disease showed 1 to be less cardiotoxic than TCA or maprotiline.[3,31-35] Mianserin did not affect peripheral NA mechanisms in humans and did not reverse the antihypertensive actions of adrenergic neuron blockers or propranolol.[36] There was no interaction between 1 and phenprocoumon in cardiac patients receiving anticoagulant therapy.[37] The precise mechanism of action of 1 is unclear, but like amitriptyline it is a central 5-HT antagonist[38,39] and blocks presynaptic alpha-adrenoceptors.[40,41] It may also block postsynaptic alpha-adrenoceptors and inhibit NA uptake at high doses,[42] but the roles of these activities in the antidepressant action of 1 have yet to be defined.

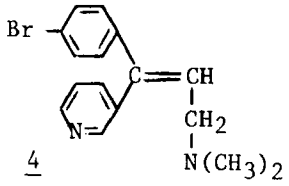
The cardiac safety of nomifensin was established by measurements of cardiac conduction in depressed patients receiving therapeutic doses over 3 weeks, and it was less toxic than doxepin or amitriptyline in isolated hearts.[43] Nomifensin inhibited NA uptake in the same way as TCA but its principal actions may be on dopaminergic (DA) systems, both as an uptake inhibitor and as an agonist.[5] The metabolites (2b, R_1=OH, R_2=H; 2c, R_1=OCH_3, R_2=OH; 2d, R_1=OH, R_2=OCH_3) inhibit the uptake of DA and 5-HT, but another potential metabolite, the catechol (2e, R_1=R_2=OH) is a potent DA agonist in behavioral and neurochemical studies.[44,45] However, the beneficial effects of 2a in parkinsonism may be due to its improvement of depressive symptoms.[46]

Trazodone (3) is another non-TCA claimed to produce minimal side-effects.[10] It showed a complex pharmacological profile in animals, including tranquilizing and antihypertensive properties, with some selectivity towards inhibition of 5-HT uptake.[6] Like mianserin and doxepin it acted at low doses as a central 5-HT antagonist, but at higher doses it was agonistic. Trazodone depressed prolactin and growth hormone secretion in humans, suggesting a possible DA-like action which may explain its beneficial use in parkinsonism.[10] In clinical trials in depression, 3 had a broad spectrum of activity, combining rapid anxiolytic effects with a slower antidepressant action comparable to various TCA and superior to placebo.[10]

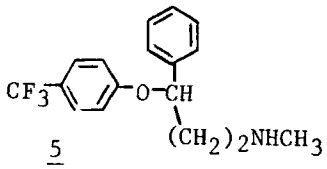
Antidepressants and 5-HT - Because of the possibility of reduced cardiotoxicity, interest was maintained in 1978 in compounds which are selective inhibitors of 5-HT uptake, many of which were reported in Volume 13 of this series[21] and elsewhere.[6] Open clinical studies of zimelidine (4) showed good antidepressant effects after doses of 150 mg daily.[47] The claimed rapid onset of action of 4 was not confirmed in the first double-blind trial in hospitalized depressives given single bedtime doses of 4 or amitriptyline for 4 weeks.[48] No difference in efficacy emerged, but side-effects were fewer with 4. A cardiovascular study showed 4 and mianserin

to be safer drugs in depressed patients than TCA, the only effects of 4 being to slow heart rate and increase QT_c interval.[33]

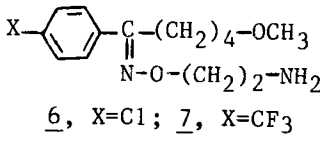
Fluoxetine (5) inhibited 5-HT uptake into platelets and also reduced platelet 5-HT content after chronic administration to healthy volunteers.[49] This dose had no effect on the usual pressor responses to NA or tyramine



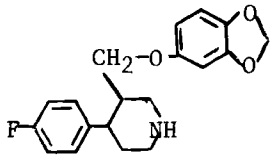
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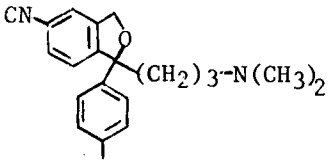
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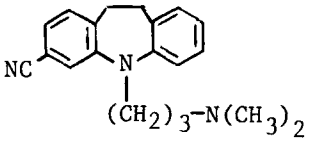
6, X=Cl; 7, X=CF_3



8



9



10

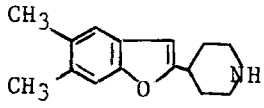
injection. Inhibition of 5-HT uptake was correlated with plasma levels of 5, and plasma from treated subjects also inhibited 5-HT uptake in platelets from untreated subjects. Fluoxetine was well absorbed and reached peak plasma levels and steady-state in 6 and 12 hours, respectively.[49] It suppressed REM sleep in cats in a dose-dependent way, an effect which was potentiated by 5-HTP.[50] Fluoxetine did not block arecoline-induced EEG desynchronization in cats.[50]

Clovoxamine (6) is the chloro analog of fluvoxamine (7), which has been shown to have antidepressant effects in humans.[21] Open clinical studies with 6 showed rapid and persistent benefit in endogenous depression without unwanted side-effects.[51] The animal pharmacological profile of 6 was similar to 7 - antagonism of reserpine effects, potentiation of 5-HTP, potent and selective inhibition of 5-HT uptake, and no anticholinergic or cardiotoxic properties.[52] Paroxetine (8), an analog of femoxetine, was more potent and longer-lasting as a 5-HT uptake inhibitor than chlorimipramine. Oral administration to rats produced long-lasting potentiation of the effects of 5-HTP.[53]

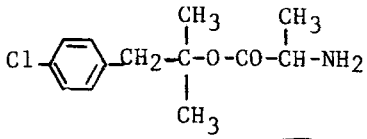
Citalopram (9) was about 2-8 times more potent *in vivo* than chlorimipramine against 5-HT uptake in rat brain or blood platelets but had essentially no effect on uptake of DA or NA.[54] *In vitro*, the two drugs were equipotent and were competitive inhibitors of 5-HT uptake but about 20-35 times more potent than imipramine or amitriptyline. The citalopram metabolites were also active. In healthy subjects, 9 had a long plasma half-life of 1 to 2 1/2 days.[55] Another nitrile, but derived from imipramine, Ro 11-2465 (10), was one of the most potent and selective inhibitors of 5-HT uptake *in vivo* and *in vitro*, being up to 20 times more potent than chlorimipramine. 5-HT content in platelets was drastically reduced in rats given 10 for 4 days.[56] Compound 10 was 30 times more potent than chlorimipramine in potentiating 5-HTP behavioral syndromes in mice.[56]

Other selective inhibitors of 5-HT include CGP 6085A (11), which has

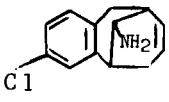
an oral ED_{50} in rats of between 1 and 4 mg/kg.[57] NA uptake was unaffected at doses up to 1000 mg/kg.[58] Like other compounds, such as fluoxetine and chlorimipramine, 11 was a more potent inhibitor of 5-HT uptake in the brain than in platelets. Alaproclate (12) was a weak competitive inhibitor of 5-HT uptake, being less potent than chlorimipramine *in vivo* and *in vitro*, but exhibiting a much greater separation of 5-HT and NA uptake effects.[59]



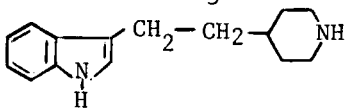
11



12



13



14

Alaproclate potentiated 5-HTP syndromes in animals and had no anticholinergic effects. Its proposed pharmacophore also accommodated Org 6582 (13). Compound 13 exerted significant inhibition of 5-HT uptake for at least 48 hours after administration to rats.[60] Competitive inhibition by 13 occurred *in vitro* at concentrations that were substantially less than those required for inhibition of DA or NA uptake, and it was more potent *in vivo* than fluoxetine and chlorimipramine.[60]

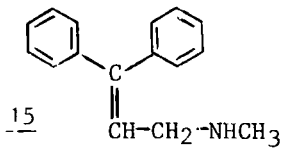
LM 5008 (14) was the most potent and selective inhibitor of 5-HT uptake of a series of indole derivatives.[61] The duration of action and potency *in vivo* were similar to chlorimipramine. LM 5008 also inhibited platelet 5-HT uptake in rats and decreased platelet 5-HT content after single or repeated dosings.[62] Open clinical trials in 40 depressed patients showed a strong and rapid antidepressant effect associated with pronounced inhibition of 5-HT uptake.[63] 7,N,N-Trimethyltryptamine selectively inhibited 5-HT uptake into rat brain synaptosomes without affecting the uptake of tryptophan, DA or NA.[64]

5-HT may be involved in the mechanism of action of antidepressants which block central 5-HT receptors at doses below those causing inhibition of 5-HT and NA uptake.[38,39] Some TCA strongly and competitively inhibit 5-HT uptake,[65] and activity may be higher in the brain than in platelets.[58] During clinical improvement some TCA, particularly chlorimipramine, lowered the rate of 5-HT transport into platelets (Vmax), which is already reduced in depression, and increased Km values.[65] Mianserin normalized Vmax concomitant with clinical improvement.[3]

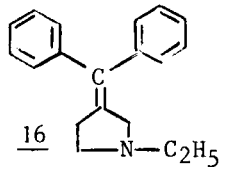
Tricyclics and Analogs - TCA, old and new, were the subject of continuing investigation. Dothiepin, a sulphur isostere of doxepin, is a typical TCA-like inhibitor of both NA and 5-HT uptake.[7] Double-blind studies showed dothiepin to be similar in efficacy to TCA after divided or single daily doses, with fewer anticholinergic effects and good tolerance in the elderly. A pharmacokinetic study showed a significant correlation between clinical response to doxepin and plasma steady-state levels of both it and its desmethyl metabolite.[66] Lofepramine, an N-(p-chlorobenzoyl) derivative of desmethyl imipramine, had reduced anticholinergic activity in healthy subjects[67] and lower cardiotoxicity in animals.[68] Double-blind trials in depression failed to reveal any other advantages over imipramine or other TCA.[69,70] Amoxapine had antidepressant and neuroleptic effects in animals,

but clinical trials showed little difference between it and imipramine and anticholinergic effects were common.[71] Amitriptyline-N-oxide differed from amitriptyline in animals only by its decreased peripheral anticholinergic activity and better gastric tolerance.[72] Clinical trials showed antidepressant activity together with anxiolytic, sedative and anticholinergic properties. Rapid and quantitative absorption of the N-oxide in humans was followed by hydroxylation at the 10-position, without any substantial conversion to the parent TCA, as was observed with imipramine-N-oxide.[73]

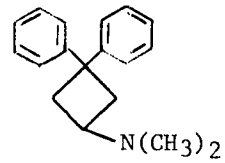
Side-effects of TCA were compared in double-blind trials in healthy subjects. Marked inhibitory effects on salivary flow declined from amitriptyline through doxepin, imipramine, nortriptyline to desipramine, paralleling subjective reports of anticholinergic side-effects and reported affinities for muscarinic receptors.[74] Similar rankings for sedation and decrements in psychometric performance were obtained in another study; BW 247 and protriptyline were inactive.[75] BW 247 (15) is a ring-opened TCA with few anticholinergic effects and a profile of mixed NA and 5-HT uptake inhibition. It is structurally related to several potential antidepressants, including AHR 1118 (16). Both 15 and 16 are effective antidepressants in man;[6] 16 showed fewer side-effects than imipramine in a



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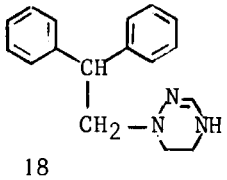


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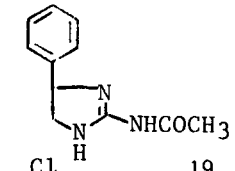
double-blind trial in depression but was somewhat less effective.[76] Compound 17 was the most potent inhibitor of NA and 5-HT uptake in a series of diphenylcycloalkanes, and it also showed some inhibition of DA uptake. In animals, 17 caused central stimulation like that of methylphenidate rather than amphetamine, and it may release DA from granular stores.[77] DL 262 (18) inhibited NA and 5-HT uptake *in vitro* and *in vivo*, and had an amitriptyline-like profile in pharmacology.[78] The imidazole analog MCPI (19) is the lead compound of a series which blocks NA but not 5-HT uptake in rat brain, does not affect MAO, and possesses weak sedative but not anticholinergic properties. MCPI was too toxic to give to man, but analogs with better tolerance are available.[79] The maprotiline analog C49802-B-Ba (20) inhibited NA uptake in rat brain and heart *in vivo* without affecting the uptake of 5-HT.[57] The new tetracyclic derivative incazane (21) was about half as potent as imipramine in pharmacological tests, with lower toxicity and little anticholinergic activity.[80] It has a slow onset of action clinically, like TCA, and combines antidepressant effects with stimulant properties.

The selective inhibition of NA uptake by viloxazine, in the absence of effects on DA or 5-HT uptake, resided in the trans-S-isomer, paralleling the reserpine reversal effects.[81] The anorectic drug mazindol was also a potent inhibitor of NA uptake,[82] like its structural relative ciclazindol (22).[83] In a double-blind trial, 22 was as effective as amitriptyline in ameliorating endogenous depression. Ciclazindol, which lacked significant anticholinergic or cardiotoxic effects, produced fewer side-effects than amitriptyline. Diverse structures included befuraline (DIV 154, 23) an

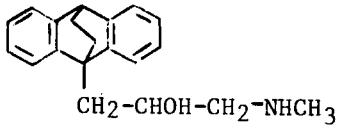
imipramine-like drug of low cardiotoxicity,[84] and EGY-475 (24) which was more potent behaviorally than imipramine and lacked cardiotoxicity and anticholinergic effects.[85] Bupropion (25) does not affect uptake or release of biogenic amines and does not block MAO.[86] Previous reports of a rapid antidepressant action for 25 were not confirmed in a multicenter double-blind trial in depression, but it was superior to placebo in the absence of marked side-effects.[87]



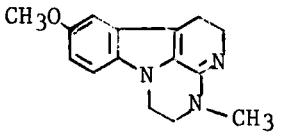
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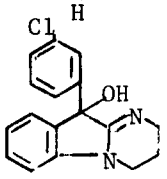
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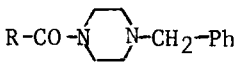
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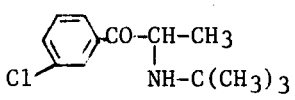


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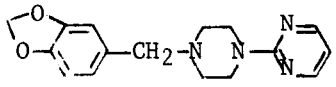


23, R = 2-benzofuranyl
24, R = 2-pyridyl

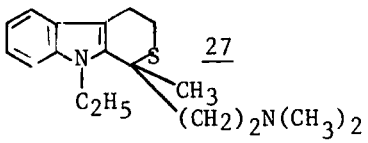
Antidepressants and DA - Antidepressants acting principally on DA systems include nomifensin (2a) and the antiparkinson drug piribedil (26). Piribedil produced moderate antidepressant effects when given in doses of 120-240 mg daily, accompanied by increased REM latency and decreased REM sleep.[88] In drug-resistant depressives, 26 produced a rapid, transient and sometimes pronounced antidepressant effect but also a consistent syndrome of anger and hostility.[89] In the rat brain, tandamine (27), a proven antidepressant in previous studies,[21] was more potent in inhibiting NA uptake than DA uptake and had virtually no effect on uptake of 5-HT.[90] The bicyclooctane derivative LR 5182 (28) blocked DA uptake in rat brain synaptosomes and also affected NA uptake at 20-fold higher concentrations.[90] This high potency and selectivity was lost in vivo, but the potent effects of 28 on DA and NA uptake suggest a possible antidepressant action.



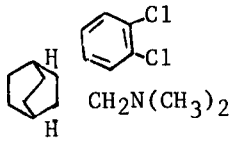
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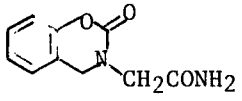
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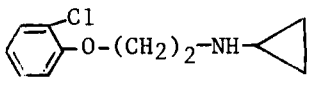
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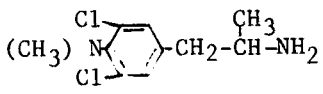
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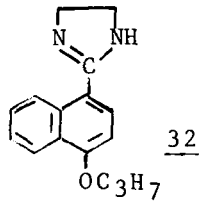
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MAOI - There was renewed interest in MAOI since their combination with TCA may be the only alternative to the use of ECT for drug-resistant patients.[91] ECT was superior to combined phenelzine and amitriptyline in severe depression,[92] but phenelzine alone was as effective as amitriptyline in depressed outpatients.[93] Caroxazone, an effective antidepressant drug (29), probably acts through reversible inhibition of the two known forms of MAO (A and B).[6]

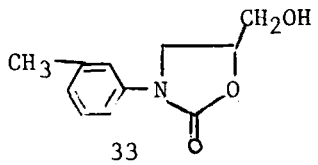
Lilly 51641 (30) was a selective and long-lasting inhibitor of MAO-A in rat brain and other tissues.[94] Compound 31 was the most active of a series of phenethylamines which had selective inhibitory effects on rat brain MAO-A and antidepressant activity in animals.[95] Anti-tetrabenazine activity was correlated with inhibition of mouse brain MAO for a series of imidazoles, and potential antidepressant activity was greatest with 32.[96] The oxazolidone toloxatone (33) acted as a specific and reversible MAO-A inhibitor in rats, was clinically effective in depression, and may act per se and through its hydroxylated metabolites.[97]



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Amino Acids - Rapid catabolism by liver tryptophan pyrrolase may be the reason for the equivocal effects of tryptophan in depression, and addition of an inhibitor of this enzyme, allopurinol, to a daily regimen of tryptophan did produce marked and safe benefit in 8 patients with endogenous depression.[98] DL-Phenylalanine produced a complete or good response in 12/20 depressed patients whereas 4 patients did not respond; depressed mood, retardation and agitation were preferentially affected.[99]

Beta-Agonists - The efficacy of beta-adrenergic agonists was confirmed.[100] Most beta-agonists show a TCA-like profile in some animal models of depression and open studies in depressed patients given intravenous salbutamol for 10 days demonstrated a rapid antidepressant effect.[100] No anticholinergic effects or orthostatic hypotension were observed, but tachycardia was dose-limiting and extrapyramidal signs were common. A controlled trial in 20 severely depressed patients showed a more rapid and effective response to salbutamol 6 mg daily than to chlorimipramine 150 mg daily, both given intravenously.[101] There are no reports of the effects of other beta-agonists in depression.

Peptides - Brain peptides and their analogs may offer the prospect of a rapidly acting antidepressant.[102,103] Beta-endorphin, an opiate-like fragment of the much longer pituitary peptide beta-lipotropin, showed antidepressant effects in pilot trials following intravenous injection.[104] The biphasic pattern of response to melanocyte-stimulating-hormone releasing factor I (MIF-I), H-Pro-Leu-Gly-NH_2,[21] was confirmed in a double-blind trial. Lower doses of MIF-I were more effective than either placebo or higher doses, acting within a few days of starting treatment orally.[105] Other orally effective analogs are available.[21] After initial promising results, most studies with thyrotrophin-releasing hormone (TRH), pGlu-His-Pro-NH_2, have demonstrated minimal antidepressant effects following single or multiple doses given orally or intravenously.[102,106] Some patients with unipolar or bipolar depression or manic-depressive illness show a reduced secretion of thyroid-stimulating hormone (TSH) in response to TRH challenge, and respond to ECT but rarely to drugs.[106,107] TRH has been suggested as a useful diagnostic and prognostic tool.[107]

New Screening Methods - New animal models for depression included the

bulbectomized rat. Bilateral olfactory bulbectomy results in a syndrome of deficient learning, hyperreactivity and elevated plasma 11-hydroxycorticosteroids, all of which were blocked by pretreatment with TCA and non-TCA, including mianserin and nomifensin, but not by other classes of psychotropic agents.[108,109] The activity of Org 6582 (13), a selective inhibitor of the uptake of 5-HT, was also demonstrated in this model. The model appears to depend upon 5-HT systems in the brain, since an identical syndrome was produced by intrabulbar injection of the 5-HT neurotoxin, 5,6-dihydroxytryptamine, but not by the DA neurotoxin, 6-hydroxydopamine.[110]

The behavioral despair model forces rats or mice to swim in a narrow cylinder of water from which they cannot escape and they ultimately adopt a characteristic posture of immobility.[111] Immobility was reduced by MAOI, TCA and by new drugs like mianserin and nomifensin, as well as by ECT, REM sleep deprivation and an enriched environment. Anxiolytics did not affect immobility, neuroleptics enhanced it, and psychostimulants reduced it but at doses also causing motor stimulation.

Another new model utilized the dose-related hypothermia produced in mice by subcutaneous clonidine.[112] Chronic, but not acute pretreatment with TCA, non-TCA or MAOI, antagonized the response, as did alpha-adrenergic blockers, but not beta-blockers, CNS stimulants, anxiolytics, anticholinergics, analgesics, or selective inhibitors of 5-HT uptake. Chronic imipramine treatment blocked clonidine hypothermia and antagonized clonidine-induced slowing of NA turnover in rat brain, but it did not alter other pharmacological effects of clonidine. A common underlying mechanism of antidepressants may be to alter alpha-adrenoreceptor sensitivity possibly by a presynaptic mechanism.[112]

Reserpine-induced ptosis in the rabbit was proposed as a 5-HT dependent test for antidepressant activity.[113] Stronger antagonism to ptosis was shown by TCA containing a tertiary amino than a secondary amino moiety, and ptosis was also blocked by selective 5-HT uptake inhibitors, 5-HTP and 5-HT-like drugs, including the new antidepressant LM 5008 (14). MAOI were also effective. Anxiolytics and neuroleptics did not reverse ptosis, nor did TCA acting principally on NA mechanisms. Nevertheless, doubts continue to be cast upon the relevance of models based upon reserpine-like drugs to the therapeutic action of antidepressants. Thus, reversal of tetrabenazine effects, also a reserpine-like drug, appeared to produce a pattern of stereotyped behavior quite distinct from the behavior of normal control animals.[114]

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Chapter 2. Antipsychotic Agents and Dopamine Agonists

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The term schizophrenia, far from implying a homogeneous syndrome, embraces clinical manifestations ranging from catatonic withdrawal to florid paranoia, whose etiologies are unknown and may not be identical.[1,2] There are no laboratory criteria for diagnosis of the disorder, and the subjective criteria are sometimes controversial.[3] Thus it may not be surprising that agreement between diagnosticians has been generally less than 60%[4] and that wide variations in course and outcome are seen. Such uncertainties have encumbered the search for characteristic neurochemical lesions. Moreover, investigators must anticipate that the complexity of the brain's interneural connections will make the crucial distinction between primary causative lesions and secondary reactive ones very difficult. Thus reports of biochemical abnormalities in schizophrenia span a confusing spectrum from prostaglandin deficiency[5,6] to errors in neurotransmitter metabolism.[7,8] Nevertheless, it is encouraging that neuroleptic (antipsychotic) drugs are clearly effective in symptomatic treatment of schizophrenia.[9,10] Among investigators in this field, the working hypothesis which currently enjoys the widest popularity is the dopamine (DA) hypothesis of schizophrenia: since virtually all known antipsychotic drugs block DA activity and since no other common denominator has yet been found, excess central dopaminergic activity may be present in the disorder[11,12] Therefore, this review begins with recent discoveries about DA receptors.

Dopamine Receptors. Since the identification of DA receptors at the biochemical level, using both adenylate cyclase and radioligand binding systems, it has become clear that a heterogeneity of DA receptors exists. One distinction which can be made is between DA ligand binding sites which are, or are not, linked to adenylate cyclase.[13,20] This point can be well illustrated by characteristics of receptors in the striatum and nigrostriatal pathway. Kainic acid injection into the striatum destroys striatal neurons on which postsynaptic DA receptors are located while sparing axons which pass through this region and terminals which end within it.[14] Such treatment produces a decrease of about 40% in ^{3}H-neuroleptic binding while destroying virtually all of the DA-sensitive adenylate cyclase activity.[14-16] This shows that about 60% of the binding sites are not cyclase-linked. Most of the remaining antagonist binding sites are lost following ablation of the cerebral cortex, indicating that they are located on axons and terminals of the cortico-striatal projections.[15,17] In addition, it has now been shown in vitro that agonist binding to sites linked to adenylate cyclase is modulated by guanyl nucleotides,[18,19] whereas binding of antagonists is not. However, after removing the portion of striatal binding sites sensitive to kainic acid lesion, the sensitivity of the remaining sites to guanyl nucleotide modulation is completely lost.[20] This implies that the receptors removed by kainic acid are linked to adenylate cyclase, whereas those remaining (on axons and terminals) are not.

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Kebabian and Calne have lately organized the available evidence into a coherent argument for classification of DA receptors as D-1 or D-2, according to whether or not they are linked to adenylate cyclase.[13] Various criteria for this classification are listed in Table 1.

Table 1. DA receptor classification criteria (adapted from ref. 13)

Receptor type		D-1 (cyclase-linked)	D-2 (not cyclase-linked)
Prototypical example		Bovine parathyroid	Pituitary mammotroph
Effects of:	DA	Agonist (μM range)	Agonist (nM range)
	APO	partial agonist or antagonist	Agonist (nM)
	Ergots	Potent antagonist (nM) or weak agonist (μM)	Agonist (nM)
Selective antagonist		None known as yet	Sulpiride
Selective ^{3}H-ligand		cis-Flupenthixol	Dihydroergocriptine

The bovine parathyroid gland is a tissue with a relatively homogeneous population of D-1 (cyclase-linked) receptors. In this tissue DA stimulates the accumulation of c-AMP, and this event is followed by release of parathyroid hormone from the gland.[21] Apomorphine (APO) acts as a pure antagonist in this system. It may be recalled that in the striatum APO in the μM range acts as an agonist in stimulation of adenylate cyclase and behaves as an antagonist at higher concentrations.[22] It appears that haloperidol and the other classical neuroleptics block both types of DA receptors.

Prolactin secretion from the anterior pituitary is modulated via DA receptors located on the mammotrophs.[23,24] Much evidence implies that these DA receptors are not linked to adenylate cyclase[24,25] and hence are D-2 receptors, although one recent study using high concentrations of agonists has reported DA-sensitive adenylate cyclase activity in that tissue.[26] Pharmacological specificity of the pituitary DA response clearly distinguishes it from systems linked to adenylate cyclase. Two main classes of drugs permit this distinction. The benzamide antipsychotic sulpiride is among the most potent drugs in producing an increase in prolactin secretion, indicating that it is a potent antagonist of the pituitary DA receptor.[24] However, it is quite ineffective in blocking DA-sensitive adenylate cyclase in the striatum.[27] Such disparate results are also seen with certain ergot alkaloids such as bromocriptine. This compound is nearly inactive in stimulating adenylate cyclase[28,29] but is a very potent inhibitor of the release of prolactin, indicating that it is a potent DA agonist on the pituitary D-2 receptors.[23]

Binding data supplied by Seeman and his colleagues have indicated that in the striatum low concentrations of APO bind primarily to presynaptic receptors while low concentrations of neuroleptics (e.g., haloperidol) bind preferentially to postsynaptic receptors.[30] Thus injection of 6-OH-DA into the substantia nigra, which destroys presynaptic nerve terminals but spares postsynaptic neurons, decreases the binding of ^{3}H-APO and increases the binding of ^{3}H-haloperidol.[30] However, it must be noted here that Creese and Snyder have reported that a similar lesion produces increases in both ^{3}H-APO and ^{3}H-spiroperidol binding.[20] The technical differences underlying this discrepancy are not yet known. The increased

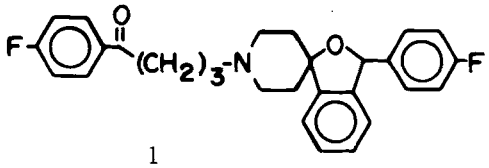
binding of antagonists is due to increased numbers of DA receptors and is a model for the postsynaptic dopaminergic supersensitivity that occurs in Parkinson's disease.[31] In the past year such drug- or disease-induced supersensitivity has been studied by many authors.[32-35] Two papers refer to the effects of lithium, a well known treatment for manic-depressive illness, in such models. Rats treated with haloperidol and lithium salts together did not develop the supersentivity which resulted from haloperidol treatment alone.[36] Lithium treatment also blocked the development of presynaptic supersensitivity, as detected by electrophysiological measurements.[37]

Earlier, Lee and Seeman reported some preliminary data showing increased numbers of DA receptors in the post-mortem brains of schizophrenic patients.[38] This important observation supported the possibility that such receptor supersensitivity underlies the etiology of schizophrenia. Those authors have now reported an expanded study showing that ^{3}H-APO binding (presynaptic) was normal in schizophrenic brains whereas ^{3}H-haloperidol binding (postsynaptic) was significantly elevated in the caudate and putamen.[39] The trend was the same in the nucleus accumbens, but in that case the sample size was very small. Owen, et al., have also reported significantly increased levels of ^{3}H-spiroperidol binding in all three of these nuclei in post-mortem schizophrenic brains, including those from seven patients who had not been medicated for at least a year before death.[40] However, Mackay, et al., detected no abnormality in the ^{3}H-spiroperidol binding in samples of nucleus accumbens from the brain of 26 schizophrenics.[41] Because of the probable importance of limbic areas such as the nucleus accumbens in schizophrenia,[42] the question of whether or not increased receptor levels are somehow responsible for the pathogenesis of this disease must still remain an open question.

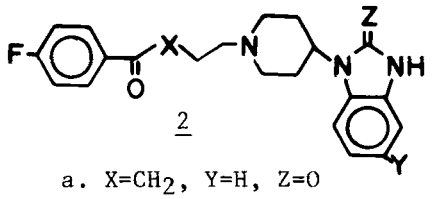
Antipsychotic Drugs. It is a corollary of the DA hypothesis of schizophrenia that pharmacological screening methods for antipsychotics are usually designed to discover DA blocking agents. Neuroleptics typically increase DA turnover, elevate prolactin levels, compete with DA receptor ligands for membrane binding sites, induce catalepsy and block conditioned avoidance responding (CAR) in trained animals. It is widely believed that extrapyramidal side effects (EPSE) are low in antipsychotic drugs with central anticholinergic properties[43,44] and that sedative and hypotensive side effects may result from α-adrenergic blockade.[44] Newer behavioral models purport to discriminate between drugs with high liability to EPSE and those with low liability. Thus Ljungberg and Ungerstedt have shown, in an APO-induced stereotypy model (rats), that classical neuroleptics (high EPSE) predominantly antagonize the compulsive gnawing while non-cataleptic neuroleptics (low EPSE) block primarily the hypermotility.[45] Prediction of EPSE has also been suggested in an APO-induced mouse climbing model.[46] A phenylethylamine-induced stereotypy model for schizophrenia[47] acquires additional significance from reports that increased levels of this amine occur in some psychotic states.[48,49]

A survey of potential new neuroleptics reported during the year shows that the large majority are variations on well-known drug classes. A Hoechst-Roussel group has reported a group of 9 new butyrophenones, of which 1 is the most interesting.[50] It is orally effective in blocking

CAR in monkeys (0.5 x chlorpromazine) but has little effect on APO- or amphetamine-induced behaviors, a combination which was expected to predict effectiveness with freedom from EPSE. A group of 29 butyrophenones generally related to benperidol (2a) has been reported[51], of which 2b is the most promising. It is very effective orally (10 x haloperidol) at blocking drug-induced stereotypies with less propensity to induce catalepsy. Halopemide (2c)[52] is a benzamide which is otherwise closely related to the butyrophenone 2a. It is a potent inhibitor of APO-induced emesis[53] and increases prolactin secretion in cultured pituitary mammotrophs.[54] A preliminary clinical report indicates that it produces low EPSE.[52] A large series of papers[55] on bromperidol (3a), which is closely related to haloperidol(3b), document the clinical efficacy,[56] behavioral



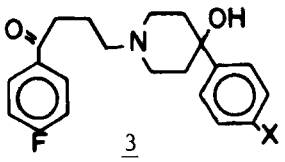
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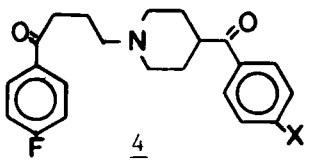
a. X=CH_2, Y=H, Z=O
b. X=CH_2, Y=H, Z=S
c. X=NH, Y=Cl, Z=O

and biochemical pharmacology,[57] neuroendocrine effects,[58] and pharmacokinetics[59,60] of this drug. Cloroperone (AHR 6134; 4a) is the chloro analog of lenperone (4b). A summary of the pharmacology and preliminary anti-schizophrenic activity of 4a has appeared.[61] Clopimozide (5a), the chloro derivative of pimozide (5b), appears to be clinically effective and long-acting without excessive side effects.[62]



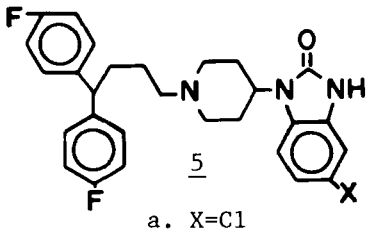
3

a. X=Br
b. X=Cl



4

a. X=Cl
b. X=F

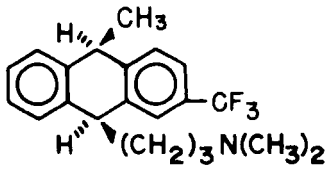


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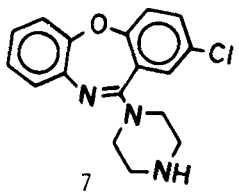
a. X=Cl
b. X=H

Patients may occasionally manifest symptoms of both depression and schizophrenia.[63] Some recent compounds are active against models of both syndromes and might thus obviate problems of multi-drug interactions. An SK&F group has reported a thorough comparison of the pharmacology of fluotracen (SKF 28175; 6) to that of standard antipsychotics and antidepressants and has found it to possess both types of activity.[64] Interestingly, removal of the 10-methyl group greatly enhances the neuroleptic effects and abolishes the antidepressant effects, while addition of a second 10-methyl group removes both types of activity. Amoxapine (7), which was previously known as a clinically effective antidepressant,[65] has now been shown by a Lederle group to have significant neuroleptic activity in a number of animal models.[66] Finally, the antidepressant trebenzomine (CI 686; 8)[67] is reported to exhibit clinical antipsychotic efficacy similar to that of chlorpromazine.[68] Its mode of action does

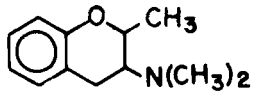
not appear to be dopaminergic, as it does not increase DA turnover, stimulate prolactin secretion, or compete for neuroleptic binding sites.[68]



6

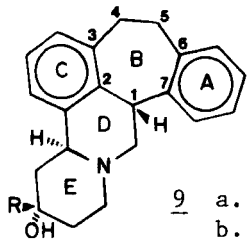


7



8

Several papers have now appeared from Ayerst groups detailing the effects of modifications to the structure of the clinically effective[69] neuroleptic butaclamol (9a). Because transposition of chlorine groups can eliminate cataleptic side effects from other tricyclic neuroleptics (e.g., octoclothepin→doclothepin), a series of chloro derivatives of the butaclamol analog 9b was made.[70] However, this produced only compounds which were inactive or retained butaclamol-like activity.[70,71] They also made a series of 15 analogs (9c) in which the t-butyl group was replaced by aryl groups.[72] This produced a variable pattern of in vivo neuroleptic activities, but generally enhanced the adrenolytic liabilities. By making a series of modifications on ring E, they concluded that the t-butyl but not the hydroxyl group is required for neuroleptic activity.[73] They also found that ring A of 9a can be moved from the 6,7-position to the 5,6-position without reduction of activity.[74] Based on geometrical analysis of these compounds, they devised a 3-dimensional model of the receptor binding site which includes a primary nitrogen binding site, a naphthalene-sized primary aromatic binding site, and an accessory binding site for the t-butyl group.[73,74] A Janssen group has also attempted to use the rigid structure of 9b to infer pharmacologically relevant conformations of more flexible neuroleptics.[75] Seeman, et al., have noted that the stereospecificity of binding to striatal sites is higher for the enantiomers of 9a than for any of 7 other resolved neuroleptic pairs, probably reflecting in part its rigid structure.[76]



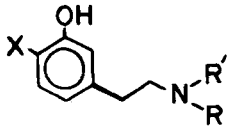
9 a. R=t-Bu
b. R=i-Pr
c. R=Ar

More data have accumulated on the interesting benzamide group of antipsychotics, of which sulpiride is the best known. It has now been shown in studies of behavior,[77] vasodilation,[78] and binding[17] that neuroleptic activity resides primarily in the (-)-S enantiomer, whose absolute configuration was determined by chiral synthesis.[17] Costall, et al., compared a group of benzamides to a group of conventional neuroleptics for their abilities to differentially antagonize some behavioral models which are believed to have different cerebral substrates, i.e., mesolimbic vs. striatal DA systems.[79] Some benzamides have also been studied for their ability to antagonize DA-induced renal vasodilation, in which model the order of potencies is not the same as that seen in the CNS.[80] Jenner, et al.,[81] have concluded that for a group of 5 benzamide drugs there is little correllation between their clinical characteristics and their activ-

ities in behavioral and biochemical models of DA antagonism.

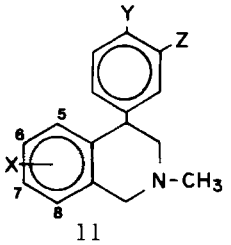
Several discussions of the relation of opioid peptides to psychosis have appeared.[82-84] Such studies raise the possibility that novel antipsychotic drugs could be based on the structure of endogenous peptides.[85] DeWied, et al., have reported that a peptide corresponding to sequence 62-77 of β-lipotropin (i.e., des-tyr^1-γ-endorphin) exhibits neuroleptic activity in behavioral models.[86] Limited antipsychotic activity of this peptide has now been reported in humans (i.m. injection).[87] However, it has also been reported that this peptide does not compete with ^{3}H-neuroleptics for binding sites in any brain areas.[88] A year ago there was a provocative report by Palmour and Ervin that the CSF of schizophrenics contains a previously unknown peptide and that this molecule can be removed by hemodialysis.[89] This would provide a simple explanation for the controversial observations that schizophrenic symptoms are alleviated by hemodialysis,[90,91] but no further data concerning the existence of this endogenous psychotogen have yet appeared.

Dopamine Agonists. During the past year a number of new modifications of the structure of DA were reported. It is notable that the effects of structure on activity in a series of compounds 10 in the CNS do not parallel those in the periphery. Compound 10a was the most potent in inhibiting the cardioaccelerator nerve in the cat, while only 10b showed any central emetic activity (0.07 x APO).[92] Compound 10b is of interest as a DA agonist in the renal vascular bed because, although it is much weaker than DA (20 x), it exhibits no α-adrenergic activity.[93] In a larger series of compounds 10, all were found to be much weaker agonists than APO in animals with unilateral cerebral lesions.[94] Compound 10c was selected for more detailed behavioral and metabolic study as a potential anti-parkinson drug.[95] Wikstrom, et al.,[96] have made the very interesting observation that the phenol 10d, although only 0.1x as potent as APO in decreasing DOPA accumulation in rat brain, is actually 10x more potent than the corresponding catechol 10b in the same model. They saw the same rank order in stimulation of motor activity. It has been reported that aromatic fluorination does not substantially alter the activity of DA at vascular receptors.[97] There are two reports in which the conformation of the ethylamine side chain of DA was constrained by incorporation into cyclopropane[98] or cyclobutane[99] rings, but the best compounds were much weaker than DA.



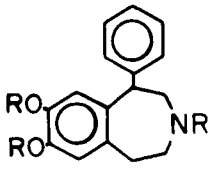
10

a. X=OH, R=R'=Me
b. X=OH, R=R'=Pr
c. X=OH, R=Pr, R'=Bu
d. X=H, R=R'=Pr



11

a. X=8-NH_2, Y=Z=H
b. X=8-NH_2, Y=OH, Z=H
c. X=8-NH_2, Y=Z=OH
d. X=7-OCH_3, Y=Z=Cl

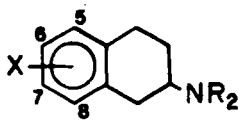


12

a. R=H
b. R=CH_3

Nomifensin (11a) is an antidepressant which produces significant DA-mimetic motor effects in animal models.[100] It has now been shown that the catechol analog 11c is a potent agonist (0.25-0.50 x DA) on the adenylate cyclase from both striatum and nucleus accumbens, whereas 11a and 11b are inactive.[101] Another group has shown that 11c is potent in inducing stereotyped behavior upon injection into the nucleus accumbens.[102] Because this effect was blocked by α-methyltyrosine pretreatment, a presynaptic mode of action was inferred. The related compound 11d has shown limited clinical efficacy in the treatment of Parkinson's disease.[103]

SK&F groups have reported that 12a (SKF 38393) is a new dopaminergic agent with a very unusual profile of activity. In the dog (i.v.) it specifically dilates the renal vascular bed without increasing cardiac output or arterial blood pressure,[104] apparently by a direct agonist effect on vascular DA receptors. In the CNS 12a is a particularly potent partial agonist in the striatal adenylate cyclase preparation and is an agonist in rats with unilateral lesions in the substantia nigra.[105] However, it fails to produce stereotypy in normal rats, is not emetic, and does not affect prolactin levels or DA turnover.[105] The unusual profile of 12a may be partially explained by considering it an agonist selective for D-1 receptors within the classification scheme discussed above. If so, it may become an important pharmacological tool. Interestingly, the dimethyl ether 12b has clinical utility as an anti-aggressive drug.[106]

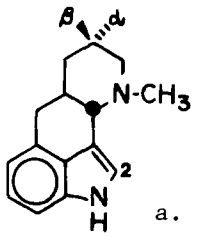


13 a. X=5,6-$(OH)_2$, R=Pr
b. X=6,7-$(OH)_2$, R=H
c. X=5-OH, R=Pr

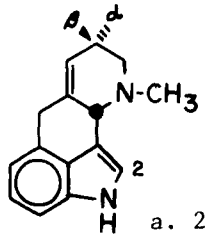
Many new data have appeared about the dopaminergic properties of 2-aminotetralins. Eighteen of these were evaluated for inhibition of ^{3}H-haloperidol binding and stimulation of striatal adenylate cyclase, and these data were compared with *in vivo* behavioral data.[107] It was noted in particular that 13a is far more potent (150 x DA) than any other known DA agonist in displacing the antagonist ^{3}H-haloperidol. It is also a potent agonist (1 x DA) in stimulating adenylate cyclase[107] and has been extensively studied *in vivo* as an agonist in behavioral models.[108-110] However, the best known compound in this series is 13b, also known as ADTN.[111,112] This compound is particularly useful for defining the agonist-specific component of ligand binding in DA receptor studies,[113] and when tritiated is a useful ligand in its own right.[114,115] Horn, *et al.*, reported that the 0,0'-dibenzoyl ester of 13b is an effective pro-drug with CNS activity because it penetrates the blood brain barrier, whereas the free catechol does not.[116] A Burroughs Wellcome group has now resolved 13b and has determined the absolute configurations of the enantiomers of 13b and 13c by degradation.[117] Studies of binding,[117] inhibition of prolactin secretion,[118] stimulation of adenylate cyclase,[119] and renal vasodilation[78] all confirm that the more active enantiomer is (+)-2R-13b. This corresponds to an absolute configuration which is opposite to those of the more active enantiomers of 13c and APO. This observation was used to form a hypothesis about the mode of binding of such agonists to DA receptors, in which the required configuration at the chiral center is controlled by the particular location of a hydroxyl group "meta" to the aminoethyl fragment.[117] This proposal may account

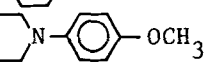
for the inactivities of iso-APO and a related structure[120] which was recently reported.

Ergots. The ergot alkaloids have generally complex pharmacological profiles,[28,121,122] but some with DA agonist properties are therapeutically useful. The best studied of these are lergotrile (14a) and bromocriptine (CB 154; 15a),[29,122-124] which are effective in treatment of Parkinson's disease.[125,126] Bromocriptine is used to treat excessive secretion of prolactin (e.g., galactorrhea) and growth hormone (acromegaly).[127] It is also an effective antihypertensive agent,[128,129] though its locus of action is unclear.



14 a. 2-Cl, (β)-CH_2CN
b. (α)-$NHSO_2N(CH_3)_2$
c. (α)-CH_2CN



15 a. 2-Br, (β)-peptide
b. (β)-CH_2-S- 
c. (β)-CH_2-N  -OCH_3

The dopaminergic activities of some newer ergolines (14) and ergolenes (15) have been studied. Compound 14b (CH 29-717) is potent (10 x 15a) in suppression of hyperprolactinemia in rats.[130] In behavioral and biochemical models 14c (CM 29-712) is generally bromocriptine-like in its DA agonist effects.[131,132] The ergolene 15b (CF 25-397) induces contralateral turning in rats with nigral 6-OH-DA lesions,[132] but exhibits few other central DA agonist effects.[28,121,131] An extensive study of 15c (MPME; PTR 17402) shows that in rat brain it is a DA agonist which is selective for subcortical limbic structures.[133]

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Chapter 3. Anti-Anxiety Agents, Anticonvulsants, and Sedative-Hypnotics

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Benzodiazepines and Related Compounds

The nature of the benzodiazepine receptor has been characterized further by the demonstration that a single class of binding sites exists in rat brain for a structurally diverse series of benzodiazepines.[1] The population size, as measured by the number of ^{3}H-diazepam (^{3}H-D, 1a) binding sites, was decreased by chronic treatment with flurazepam (1j).[2] Binding of ^{3}H-D and ^{3}H-flunitrazepam (1b) to their receptors was shown to occur *in vivo* as well as *in vitro*.[3] Regions of the rat brain containing the highest number of ^{3}H-D binding sites were the cerebral cortex, hippocampus, and cerebellum.[4] On a cellular level, studies on bovine cortical preparations indicate that the benzodiazepine receptor is localized largely on the astroglial plasma membrane.[5] However, studies with "nervous" mutant mice suggest that in this species the receptors also reside on cerebellar Purkinje cells.[6] Ontogenetic studies indicate that benzodiazepine receptors are already present in rat brain at birth, and reach maximal concentration one week later.[7]

Several recent studies indicate that some interaction may exist between the benzodiazepine and GABA receptors.[4,8] It has been found that GABA[9,10] or the GABA-agonist muscimol[10] cause an increase in the affinity of ^{3}H-D for its binding site which is markedly dependent on the presence of NaCl,[9,10] while the GABA-antagonist bicuculline decreased affinity. Inosine and hypoxanthine have been identified as endogenous ligands for ^{3}H-D binding sites in bovine brain,[11] as has a heat-stable protein of M.W.>15,000 isolated from rat brain.[12] The latter was found to inhibit GABA binding, and hence the benzodiazepines may enhance the effects of GABA by displacing this endogenous ligand from its receptor. Recent work in this area has been comprehensively reviewed.[13]

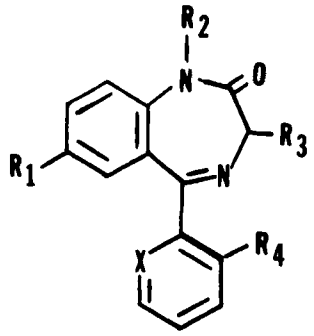
The finding that physostigmine is a highly potent antidote in acute diazepam intoxication[14] raises the possibility that benzodiazepine activity may also involve cholinergic systems.

Studies on the pharmacokinetics of flunitrazepam (1b) in man,[15] clonazepam (1h) in rhesus monkeys,[16] and triazolam (9a) in dogs[17] have appeared. The metabolism of bromazepam (1i) in rodents has been studied, and the major metabolic pathway is cleavage of the benzodiazepine ring at the N_1-C_2 and N_4-C_5 bonds with subsequent reduction and hydroxylation of the resulting primary metabolite 2-(2-amino-5-bromobenzoyl) pyridine.[18]

The subject of benzodiazepine dependence has been exhaustively reviewed,[19] and the risk factor appears to be low.

A correlation between blockade of pentylenetetrazole (leptazole)-

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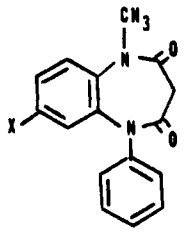


	R_1	R_2	R_3	R_4	X
1a	Cl	CH_3	H	H	CH
1b	NO_2	CH_3	H	F	CH
1c	Cl	CH_2CF_3	H	H	CH
1d	Cl	$CH_2CH_2OCH_2$-◁	H	H	CH
1e	Cl	CH_3	H	F	CH
1f	Cl	$CH_2C{\equiv}CH$	H	H	CH
1g	Cl	H	OH	Cl	CH
1h	NO_2	H	H	Cl	CH
1i	Br	H	H	H	N
1j	Cl	$CH_2CH_2N(Et)_2$	H	F	CH
1k	NO_2	H	H	H	CH
1l	Cl	CH_3	OH	H	CH
1m	Cl	H	OH	H	CH
1n	Cl	$CH_2P(O)(CH_3)_2$	H	H	CH
1o	Br	CH_3	H	Cl	CH

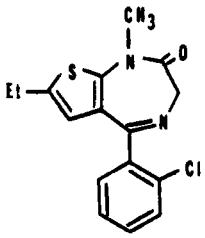
induced convulsions in mice and rate constants for the $NaBH_4$ reduction of 11 different 1,4-benzodiazepinones has been demonstrated, suggesting a possible involvement of the carbonyl group at the receptor site.[20]

Anxiolytic Agents - The animal pharmacology, metabolism, clinical pharmacokinetics, and clinical applications of lorazepam (Wy-4036 1g) in various anxiety states has been reviewed.[21] Halazepam (Sch 12041, 1c) in controlled clinical studies has been shown to be an effective anxiolytic agent at 40-600 mg/day with relatively minor side effects.[22] In a double-blind trial vs. chlordiazepoxide, clazepam (1d) at 30 mg daily was only moderately effective in decreasing anxiety.[23] ID-540 (1e) was shown to increase peak latency of the photopalpebral reflex in man, a test which may constitute a useful method for assessing potential anxiolytic activity in the clinic.[24] Pinazepam (1f) appeared to have a greater anxiolytic effect in rats than diazepam.[25] In two double-blind clinical trials, clobazam (2a) at 20-30 mg daily was found half as potent as diazepam in the relief of anxiety.[26] Its neuropharmacological profile in animals has been established,[27] and its effects on psychomotor performance in man have been studied.[28] The structurally related ORF-8063 (2b) at 66.5 mg daily was judged to be an effective antianxiety agent with energizing properties in an uncontrolled study.[29] Controlled studies in normal subjects showed clotiazepine (BAYg 5633, 3) at 10 mg to be an effective anxiolytic.[30]

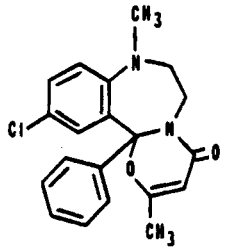
In double-blind placebo controlled studies, ketazolam (4) at about 8 mg appeared to be of significant benefit in relieving anxiety and was well-tolerated.[31] The 2,3-benzodiazepine, tofisopam (5), was claimed to exert anxiolytic effects in man without muscle-relaxant or sedative effects.[32]



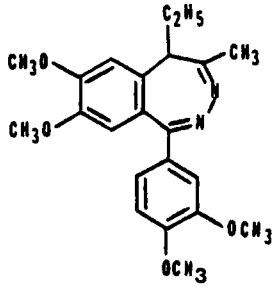
2a, X=Cl
2b, X=CF_3



3



4



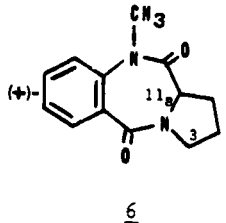
5

Although the pyrrolobenzodiazepine 6 was active in several animal tests indicative of anxiolytic activity, it was considerably less active than diazepam in preliminary tests in man. Metabolism studies suggested inactivation via hydroxylation at the 3 and 11a-positions. Attempts to increase potency and duration of action by blocking these positions with alkyl groups gave much less active compounds.[33]

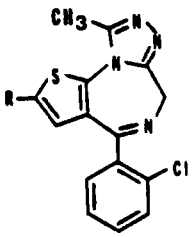
The thienodiazepine Y-7131 (AHR-3219, 7a) was found to have potent anti-anxiety, anti-convulsant, muscle-relaxant, and sedative properties in several animal species.[34] Pharmacodynamics[35] and effects of this compound on biogenic amine metabolism[36] have been described. Syntheses and SAR have also been described.[37]

Imidazobenzodiazepine 8a[38] and the related pyrrolobenzodiazepine 8b[39] were the best members of their respective series, having taming and sedative/muscle relaxant properties similar to diazepam in rodents.

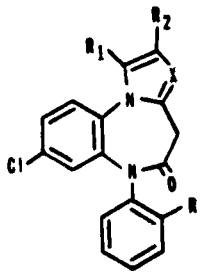
Sedative Hynotics - Sleep laboratory studies with benzodiazepines have defined the phenomenon of "rebound insomnia" which occurs on abrupt withdrawal of drug. This phenomenon seems to be associated only with the short-acting triazolam (9a), flunitrazepam (1b) and nitrazepam (1k) but not with the longer acting flurazepam (1j) or diazepam (1a).[40]



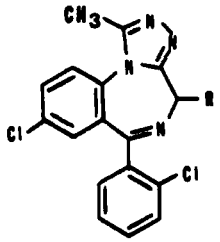
6



1a, R=C_2H_5
1b, R=Br



8a, R_1=R_3=H; R_2=C_2H_5; X=N
8b, R_1=CH_3; R_2=H; R_3=Cl; X=C-CH_3



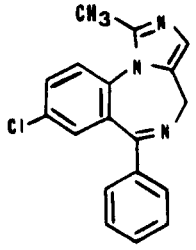
9a, R=H; X=Cl
9b, R=OH; X=Cl
9c, R=OH; X=H

Triazolam (9a)(0.125-1.0 mg) was an effective sleep-inducer in controlled studies in insomniacs,[41,42] presurgical patients,[43] and geriatrics.[44] At equieffective hypnotic doses in man, triazolam (0.5 mg) caused less performance deficits than flurazepam (30 mg) when tested the following morning.[45] An open, multi-center study indicated that temazepam (11) at 10-30 mg was an effective, well-tolerated hypnotic.[46] However, sleep laboratory studies with insomniacs indicated no effect on sleep induction or maintenance.[47]

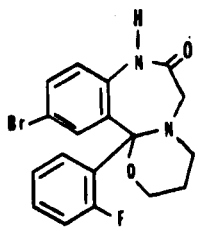
Oxazepam (1m) at 15-30 mg was judged a useful hypnotic in controlled studies in normal subjects[48] and in geriatrics.[49] Flunitrazepam (1b) at 2 mg was an effective hypnotic with few side-effects in psychiatric inpatients.[50] Controlled clinical studies indicate that fosazepam (1n) at 60 mg is an effective sedative-anxiolytic.[51] Midazolam (10) appears to be useful as an i.v. induction agent in anaesthesia.[52] WE 941 (7b) at 0.1-0.5 mg was sedative-tranquilizing in normal volunteers.[53]

The oxazolobenzodiazepine CS-430 (11) showed sedative-anxiolytic effects in animal studies.[54] A study on tissue distribution of nitrazepam (1k) in rats has appeared.[55]

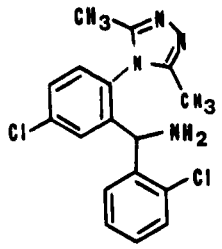
Anticonvulsants - Clobazam (2a) blocked convulsive seizures in mice and baboons.[56] The 7-bromobenzodiazepine 1o was the best of a series in protection against PTZ and maximal electroshock (MES)-induced convulsions in mice.[57]



10



11



12

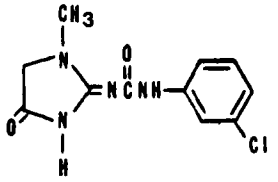
The triazole 12 derived from the corresponding triazolobenzodiazepine by fission of the C_4-N_5 bond was typical of a series of such compounds in antagonizing convulsions due to thiosemicarbazide, PTZ, or MES in mice, but not in rats.[58] The 4-hydroxylated metabolites of triazolam 9b and alprazolam 9c possess a low order of anticonvulsant activity in contrast to the hydroxylated metabolites of diazepam.[59]

Non-Benzodiazepines

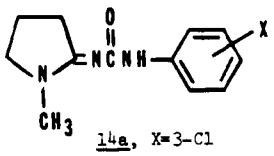
Anxiolytic Agents - The use of anxiolytics in clinical practice has been reviewed.[60] Several clinical strategies designed to distinguish anxiolytics from neuroleptics,[61] and to differentiate true anxiolysis from sedative properties[30,62] have been described. The use of animal models for the identification,[63] delineation of mechanism,[64] and clinical prognosis[65] of anxiolytic drugs has been described.

Fenobam (McN-3377, 13), which had a pharmacological profile in animals indicative of anxiolysis, showed EEG effects in man more typical of psychostimulants.[66] Of the related pyrrolidinylureas, 14a was the most interesting potential anxiolytic in evaluations in rodents.[67]

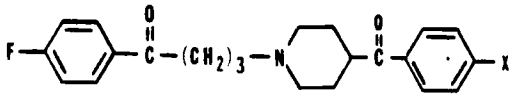
AHR-6134 (15a) at 1-8 mg,[68] lenperone (15b) at 6-40 mg,[69] brofoxine (16) at 60-150 mg,[70] and nabilone (17) at 0.5-5 mg daily[71] doses were effective anxiolytics. Sch 12679 (18) at 75-225 mg daily was effective after 2-4 weeks of administration in the treatment of anxiety neurosis.[72] Centazolone (19)[73] was found to have potent tranquillosedative, hypnotic, muscle relaxant and anticonvulsant properties in animals.[74] In normal subjects, the drug lacked undesirable side effects in single doses (40 mg) and was sedating at 20 mg.[75] The endocrine-inactive L-norgestrel (20) showed an EEG pattern typical of sedative-anxiolytics.[76]



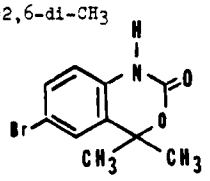
13



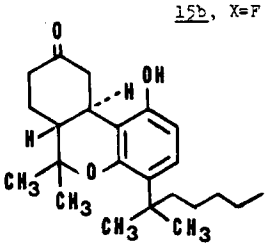
14a, X=3-Cl
14b, X=2,6-di-CH_3



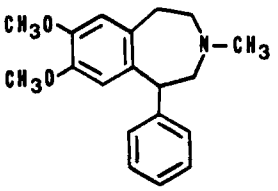
15a, X=Cl
15b, X=F



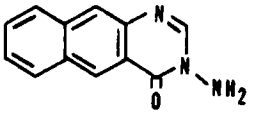
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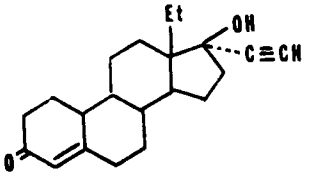
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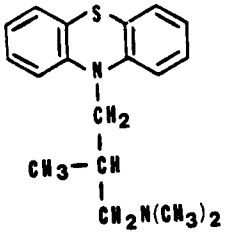
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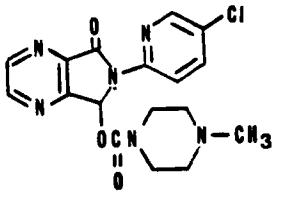
19



20

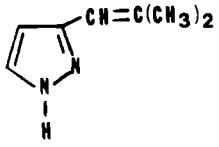


21

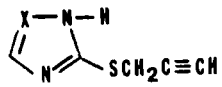


22

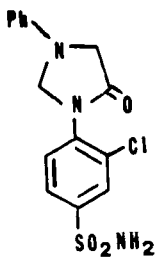
Sedative-Hypnotics - The sedative-antihistamine trimeperazine (21) at 20 mg was inferior to nitrazepam (5 mg) as a sleep inducer in a double-blind study.[77] Clinical trials indicate that zopiclone (RP-27,267, 22) is useful as a hypnotic and is as active as nitrazepam. The compound was active in a variety of animal tests with a profile similar to the benzodiazepines.[78] The pyrazole 23,[79] triazole 24a and imidazole 24b[80] all potentiated hexobarbital-induced sleep time.



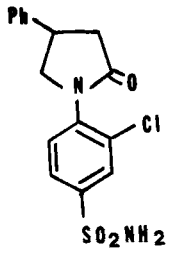
23



24a, X=N
24b, X=CH



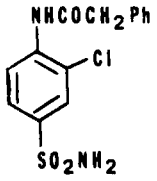
25



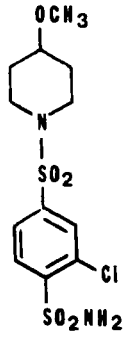
26

Anticonvulsant Agents - Compounds reported to block PTZ and/or MES-induced convulsions in mice include the benzenesulfonamide derivatives 25,[81] 26,[82] 27,[83] and 28;[84] pyranothiophenes and the related pyranothienopyrimidines, the best of which were 29[85] and 30;[86] oxadiazoles, the best of which were 31[87] and 32[88]; semicarbazide 33[89] and thiosemicarbazide 34[90]; phenothiazine 35;[91] quinazolone 36[92]; novel structures 37,[93] 38,[94] 39,[95] 40,[96] and 41[97]; and the non-nitrogen containing compounds 42,[98] 43,[99] and 44.[100]

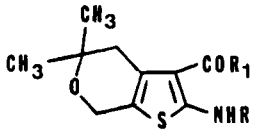
Xilobam (McN 3113, 14b) was described as a centrally acting muscle relaxant without anxiolytic or sedative properties.[101]



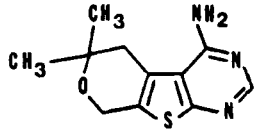
27



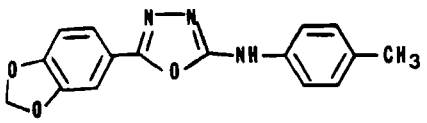
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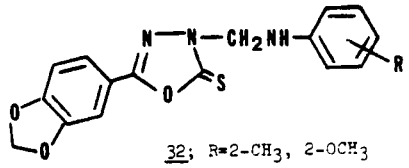
29a, R=H; R_1=m-$NO_2C_6H_4$
29b, R=$COCH_3$; R_1=OC_2H_5



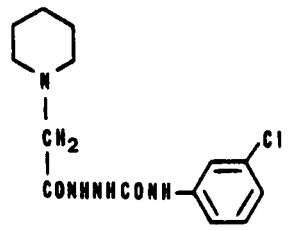
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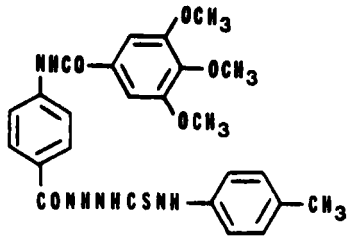
31



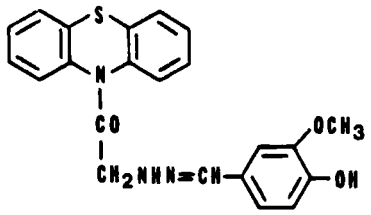
32; R=2-CH_3, 2-OCH_3



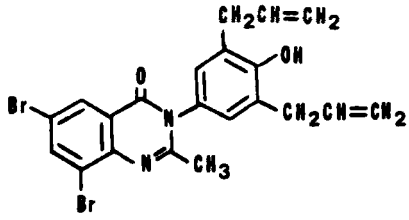
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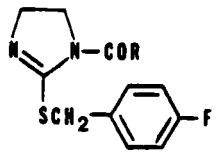
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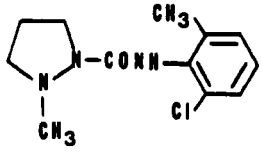
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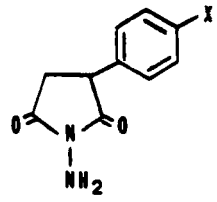
36



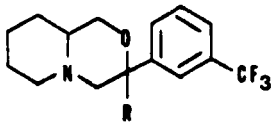
37a, R=CH_3
37b, R=OCH_3



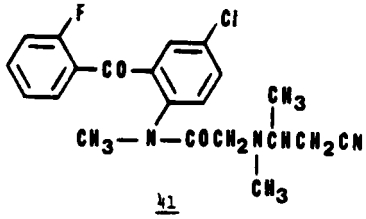
38



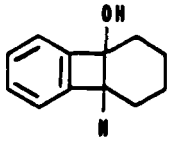
39a, X=Br
39b, X=H



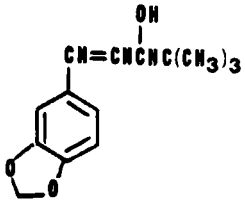
40a, R=H
40b, R=OH



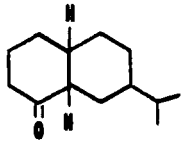
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Chapter 4. Analgetics, Endorphins and the Opioid Receptor

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Introduction - The increased intensity of research stimulated by the discovery and characterization of the endorphins has been maintained throughout 1978. Significant advances in knowledge of the biosynthesis and neurophysiology of endorphins have been made and the first reports of clinical studies have appeared. In the non-peptide field, exciting discoveries on approaches to novel narcotic antagonists have been reported. The proceedings of several symposia[1-4] have been published, as well as reviews[5-8] on research related to processes involving opioid receptors.

Endorphins

Biosynthesis - The biosynthesis of endorphins has been the subject of intensive investigation. Pulse-chase methodology has been used to show that in rats pars intermedia,[9] and mouse pituitary tumor cell line AtT20/D-16v,[10] radio-labelled amino acids are incorporated into an ACTH/endorphin precursor (31K precursor). This molecule then degrades to an endorphin-containing molecule and an ACTH-containing molecule; both β-endorphin and β-lipotropin (β-LPH) were identified in the endorphin series. Similar processes have been characterized in mouse anterior pituitary,[11] rat pituitary,[12] and human non-pituitary tumors.[13] No trace of Leu^5-β-endorphin was detected during these studies.[12,14] Studies in rhesus monkeys have shown that the breakdown of the 'stem hormone' (31K precursor) can change with age, giving rise to different relative amounts of metabolites.[15] The finding that there is a striking increase of β-endorphin in neonate (12 hrs postpartum) monkey pituitary, possibly rendering the fetus insensitive to the assault of parturition, may prove to have great physiological importance. The induction of a withdrawal-like phenomenon in fetal guinea-pig ileum upon naloxone challenge also points to a physiological role for endorphins in pregnancy.[16] Experimental evidence to support the hypothesis that the primary biosynthetic route to Met^5-enkephalin is β-LPH to β-endorphin and eventually to Met^5-enkephalin is still awaited. Recent evidence supports the existence of two separate opioid receptor systems in brain; using a combination of anatomical, biochemical and immunological methods, several groups have differentiated enkephalin neuronal systems from β-LPH/β-endorphin/ACTH positive systems.[17,18]

Metabolism - It has been shown that aminopeptidase-induced enkephalin degradation proceeds at the same rate whether or not the peptide is bound to its receptors.[19] The degradation rate, however, is a linear function of substrate concentration regardless of the degree of receptor occupancy. It is suggested that these results indicate that enkephalin binding to opioid receptors is coupled to subsequent degradation. The presence has been reported of a high-affinity peptidase in a particulate fraction of mouse striatum which cleaves Leu^5-enkephalin to yield the N-terminal tripeptide (Tyr-Gly-Gly).[20] The selective increase in the activity of this peptidase in mouse striatum after chronic morphine treatment suggests that it might be associated with enkephalinergic transmission. Cleavage of the Gly^3-Phe^4 bond of the enkephalins has also been demonstrated on incubation

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with kidney peptidyl dipeptidase (angiotensin converting enzyme).[21] The specific inhibitor SQ 14225 blocked this process. The metabolism of D-Ala^2, Met^5-enkephalinamide has been investigated in the mouse.[22] Analgesic activity (tail flick) correlated with brain concentration following i.v. administration of the pentapeptide. It has been shown that D-phenylalanine (250 mg/kg, i.p.) gives rise to significant naloxone-reversible antinociception (mouse hot plate test). It is suggested that this effect is due to the inhibition of endogenous enkephalin or endorphin metabolism.[23]

Pharmacology of Endorphins - The potencies of enkephalin analogs in inhibiting neurotransmission in the cat nictitating membrane,[24,25] and the rabbit ear artery,[24] have been investigated. The rank order of potency of most analogs on the cat nictitating membrane corresponded with that obtained on the guinea-pig ileum, while results with the rabbit ear artery gave a rank order similar to that obtained on the mouse vas deferens. It has been reported that the histamine-H_2 antagonist cimetidine at high concentrations can antagonize the actions of opiates such as Leu^5-enkephalin and morphine on the guinea-pig ileum.[26] An approach to studying the functional role of enkephalin in the nervous system using tissue cultures of mouse spinal cord neurone has been reported.[27] The results obtained suggest that Leu^5-enkephalin is capable of acting as a neurotransmitter, neurohormone or neuromodulator.

The effects of endorphins on a wide range of pharmacological and physiological processes have been investigated. The finding that endorphins induce a range of cardiovascular effects after central administration suggests their involvement in central control of cardiovascular function.[28] The reversal of endotoxin-induced hypotension by naloxone implies that endorphins may have a role in the physiology of shock.[29] The detection of large amounts of enkephalin in the gut has initiated studies of their effects on gastro-intestinal processes.[30] Both Met^5-enkephalin and morphine have been shown to increase histamine-induced gastric secretion and gastric mucosal blood flow,[31] and to inhibit induced pancreatic bicarbonate secretion in dogs.[32] The reversal of these effects by naloxone suggests a mechanism involving opioid receptors. It has been found that morphine and β-endorphin exert similar effects on the endocrine pancreas.[33] These results indicate the presence of opioid receptors on the islets of Langerhans. The observation that naloxone selectively abolishes over-eating in genetically obese mice and rats, and of elevated β-endorphin concentrations in the pituitaries of these strains, suggests a relationship between β-endorphin and obesity. Whether this effect is centrally mediated or is a consequence of interaction with gastrointestinal opioid receptors is unclear.[34] Various aspects of the endocrinology of the endorphins have been investigated. A variety of enkephalin analogs have been shown to stimulate prolactin,[35-37] and growth hormine,[35] secretion *in vivo*. The function of enkephalin in the transmission of nociceptive stimuli has received much attention. The striking similarity between the anatomical distributions of opioid receptor sites, enkephalin and substance P in brain stem and spinal cord, as well as the naloxone-reversible inhibition of substance P release from rat spinal trigeminal nucleus by a variety of opioid agonists, has led to the hypothesis that enkephalin acts as a presynaptic neuromodulator on substance P terminals.[38] This hypothesis is supported by the report that the spontaneous

firing frequency of nociceptive neurones in cat nucleus caudalis was excited by substance P and decreased by Met^5-enkephalin, and that the effects of enkephalin were in most cases blocked by naloxone.[39] Similar effects have been observed in cat spinal dorsal horn.[40] As a corollary to this hypothesis, substance P should possess analgesic properties. Recent work has shown that substance P could have a dual action on nociception;[41] at low doses substance P (1.25-5 ng/mouse, intracerebroventricularly (i.c.v.)) causes naloxone-reversible antinociception, whereas at higher doses (>50 ng/mouse i.c.v.) this effect is lost. However, at these higher doses substance P produced hyperalgesia when combined with naloxone (this effect being greater than in the naloxone control). These rather complex actions may be explained by substance P releasing endorphins at low doses, and, at higher doses, directly exciting neuronal activity in nociceptive pathways. Injection of substance P into the periaqueductal gray matter of rats produces naloxone-reversible analgesia.[42] The hypothesis that acupuncture produces analgesia through the release of endorphins has received much attention. This view has been supported by the demonstration that CXBK mice that are deficient in opioid receptors show poor electroacupuncture analgesia[43] and by the suppresion of naloxone-precipitated withdrawal symptoms in morphine-dependent mice by electroacupuncture.[44] It has been reported that rats trained to discriminate morphine from saline will respond to D-Ala^2-enkephalinamide similarly as to an internal cue of morphine.[45]

Studies in Man - Many reports have appeared on the actions of endorphins in man. The enkephalin analog FK 33-824 (1) has been used in two studies. In a single blind volunteer study, 1 (0.1-1.2 mg, i.m.) was found to be free of effects on respiratory rate and blood pressure but gave rise to a feeling of muscular 'heaviness', and in 50-60% of the subjects, 'anaphylactoid' and gastrointestinal symptoms were reported. Dose-dependent stimulatory effects on the secretion of prolactin and growth hormone were also observed; the effect on prolactin apparently was not abolished by pretreatment with nalorphine. Classical morphine symptoms such as changes in emotional behavior or nausea were not observed.[46] The hormonal and metabolic responses to 1 were measured in a double-blind trial.[47] Again the analog had a pronounced endocrine effect, raising prolactin and growth hormone levels and lowering LH, FSH and ACTH levels. Low doses of naloxone partially blocked these effects. The metabolic effects evoked by 1 were similar to those reported for morphine.

Since the discovery of the endorphins, there has been much speculation concerning their significance in psychiatric disorders.[48,49] If increased levels of endorphins are related to abnormal behavior, then it is conceivable that this behavior might be effected by narcotic antagonists. Naloxone was without effect on mood in normal human volunteers.[50] However, recent results suggest that it may attenuate symptoms in a subpopulation of patients with bipolar depression.[51] The results of a double-blind cross-over study suggest that naloxone is effective in reducing auditory hallucinations in 'some' schizophrenic patients.[103] Preliminary studies with (des-Tyr^1)-δ-endorphin (β-lipotropin 62-67) in schizophrenic patients have indicated that this material has neuroleptic activity.[52,53] Various aspects of the role of endorphins in human pain perception have been investigated. Both endorphin-like[54] and enkephalin-like[55] material have been shown to increase in the cerebrospinal fluid of patients undergoing electrical stimulation procedures for the relief of intractable pain. Although naloxone was

without effect on perception of experimentally produced pain, enhancement of clinical pain perception by naloxone in patients responding to 'placebo analgesia' suggests that this effect is produced by the action of endorphins.[56,57] However, the interpretation of these data has been criticized.[58]

H-Tyr-D-Ala-Gly-Me Phe-Met (O)-ol 1
H-Tyr-D-Ala-Gly-Phe N-MeMet-NH_2 2
H-Arg-Tyr-Gly-Gly-Phe-Met-OH 3

Endorphin Analogs - D-Ala^2, N-$MeMet^5$-enkephalinamide (2) has been found to be a potent parenteral analgesic.[59] It was 238 times as potent as normorphine in the mouse vas deferens assay, and four times as potent as morphine in the mouse hot plate assay (jump response) after subcutaneous administration. It is claimed that 2 has relatively little respiratory depressant properties or tendency to cause tolerance or physical dependence. A series of Met^5-enkephalin analogs extended at the amino terminus with amino acid residues corresponding to β-LPH have been described.[60] Using the guinea-pig ileum-myenteric plexus assay, potency was found to decrease dramatically on further extension of the β-LPH 60-65 sequence 3.

The Opioid Receptor

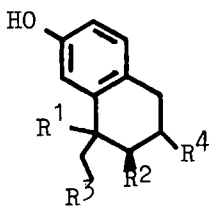
Conformation Studies of Enkephalins - Investigation of the conformation of the enkephalins and comparisons with non-peptide opiates has continued. In a review of earlier work, it was noted that several groups have assigned incorrect absolute configurations to morphine and enkephalin.[61] An X-ray crystallographic study of Leu^5-enkephalin has indicated that the peptide adopts a β-bend conformation with hydrogen bonds between the tyrosine nitrogen and the carbonyl oxygen of phenylalanine, and, between the phenylalanine nitrogen and the carbonyl oxygen of tyrosine.[62] Good correlation between the structural features of Met^5-enkephalin and a wide variety of non-peptide opioid drugs is obtained when the peptide is assigned a β-II' conformation.[63] Empirical and quantum mechanical methods have been used to identify low-energy conformations of Met^5-enkephalin and its D-ala^2 analog which may be related to rigid opioids.[64]

Receptor Models - Conformational analyses of several non-peptide opioid agonists and antagonists offers supportive evidence for the assumption that the essential difference between them arise from subtle conformational changes in the piperidine ring, leading to different interactions with the amine binding site.[65] Quantum-mechanical calculations of six oxymorphone derivatives showed that a multiplicity of low energy conformations exists for various N-substituted derivatives, possibly explaining essential differences between agonists and antagonists.[66] Experimental observation of changing agonist/antagonist ratios in some N-alkyl morphines goes some way towards supporting these studies.[67] X-ray examination of seven-membered C-ring homologs 4a-4c, and an N-positional isomer 4d, showed that the lone electron pair on the nitrogen of 4b and 4c projects away from, and that of 4a and 4d projects toward the benzene ring.[65] As these four compounds have potencies between those of morphine and codeine, it must be concluded that N-lone pair orientation does not necessarily account for all structurally induced variations of pharmacological properties.

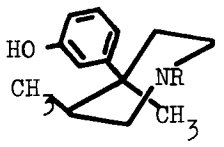
Opioid Receptor Multiplicity - The investigation of the concept of multiple opioid receptors has received further attention. Structure-activity studies with enkephalin analogs have provided support for the hypothesis that analgesia is mediated by the μ-receptor.[69] It has been found that analgesia without EEG change is observed after injection of Met^5-enkephalin into caudal midbrain periaqueductal gray matter, and that seizures and other EEG changes, without analgesia, are seen following injections into the dorsomedial thalamus. It is suggested that seizures are mediated by δ-receptors in the dorsomedial thalamus and analgesia by μ-receptors in the periaqueductal gray matter.[70] The finding that D-Ala^2 Leu^5-enkephalinamide binding sites are more numerous than those of dihydromorphine binding sites supports the hypothesis of multiple opioid receptors.[71]

Analgetics

Novel Antagonist Effects - There have been a number of very interesting developments in this area. A further study has been reported on antagonist activity in the 4-(3-hydroxyphenyl)-3-methylpiperidine series of structure 5.[72] Classical antagonist pharmacophores did not increase antagonist activity. Thus, 5, R=CH_2-c-C_3H_5, (AD_{50} 0.72 mg/kg s.c.) is a less potent antagonist than 5, R=CH_3, (AD_{50} 0.24 mg/kg s.c.) (rat tail heat against morphine), but slightly more potent in the Straub tail test. Both diastereoisomers of the propiophenone analog 5, R=$PhCOCH_2CH_2$ were antagonists, the (+)-isomer being 206 times the potency of the (-)-isomer. The racemate was without measurable narcotic agonist effect in the guinea-pig ileum, and completely antagonized the effects of morphine at 10 ng/ml. Similarly, 3-β-methyl substitution in ketobemidone, gave a partial agonist of weak antagonist character (AD_{50} 21 mg/kg (rat)). Antagonism has been detected in a fentanyl analog similarly substituted at the 3 position.[73] R 34995, (-)-6, is a very potent agonist (ED_{50} 0.0006 mg/kg (rat tail flick)), whereas the (+)-isomer, R 34994, is only a weak agonist (ED_{50} 2.2 mg/kg) which shows significant but short acting (<5 min.) antagonism of fentanyl-induced respiratory depression. R 34995, (-)-6, shows a long duration of activity, apparently due to long lasting opioid receptor binding.[74] Very potent pure antagonists are also seen in a substituted metazocine series.[75]



4



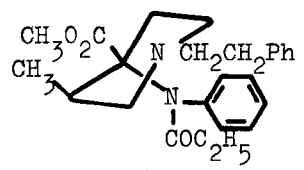
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a, $R^1 = CH_3, R^2 = H, R^3\text{-}R^4 = \text{-}CH_2CH_2N(CH_3)\text{-}$

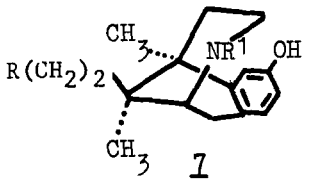
b, $R^1 = H, R^2 = H, R^3\text{-}R^4 = \text{-}CH_2N(CH_3)CH_2\text{-}$

c, $R^1 = CH_3, R^2 = CH_3, R^3\text{-}R^4 = \text{-}CH_2N(CH_3)CH_2\text{-}$

d, $R^1 = CH_3, R^2 = H, R^3\text{-}R^4 = \text{-}N(CH_3)CH_2\text{-}$



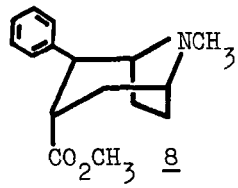
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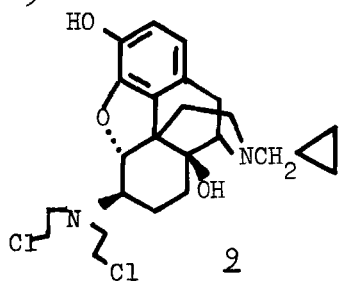
7

a, R = CO $(CH_2)_4CH_3$
b, R = CO $(CH_2)_2CH(CH_3)_2$
c, R = CO $(CH_2)_2Ph$
d, R = CO (CH_2)-c-C_5H_9
e, R = $C(CH_3)_3$, R^1 = CH_2-c-C_3H_5

$R^1 = CH_3$



8



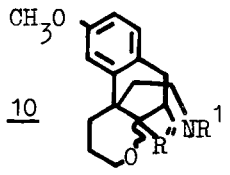
9

(±)-Win 42156, 7a, is equipotent to naloxone as a phenazocine antagonist (AD_{50} 0.008 mg/kg), the (-)-isomer being twice as potent. (-)-Win 42964, 7b, has a similar profile to buprenorphine but was slightly more potent (0.088 vs 0.11 mg/kg) in the acetylcholine test (mouse), and slightly less potent (0.026 vs 0.0043 mg/kg) in the bradykinin test (rat). (±)-Win 43632, 7c, and (±)-Win 4441, 7d, are potent pure antagonists, supported by their binding behavior, their in vitro antagonism of ethylketocyclazocine, and their inactivity in the bradykinin test. Resolution of the cyclazocine analog, 7e, of buprenorphine showed that only the (-)-series had antagonist activity, N-methyl analogs also had some antagonist character.[76] Tropanes, 8, were found to be narcotic antagonists devoid of demonstrable analgesic activities.[77]

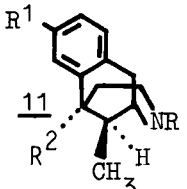
Tolerance-Dependence - Antagonists such as naloxone[78] and naltrexone[79] inhibited the development of acute dependence to morphine. Adminstration of morphine alone, or in combination with naloxone, in doses producing differing degrees of antinociception, indicates that the physiological stress leading to tolerance/dependence is proportional to the degree of analgesia experienced and not to the amount of morphine present.[78] Studies on the narcotic cue may lead to understanding of mechanisms of tolerance.[80] It is shown that the response to narcotics in rats changes with age.[81-82] Nociceptive stimulation (rat tail clip) is reported to prevent development of tolerance to fentanyl (0.04 mg/kg) given twice daily over 4 consecutive days.

Other Chemical Entities - Morphines-Morphinans - A study of the effect of morphine on castor oil induced increase in intestinal transit shows that the antidiarrheal action of morphine has both central and peripheral components.[84] (+)-Naloxone has been prepared,[85] and in rat brain binding, guinea-pig ileum, neuroblastoma-x-glioma, and hybrid cell adenylate cyclase assays, it had no more than 10^{-3} - 10^{-4} x the activity of (-)-naloxone, and can therefore be used to test the stereospecificity of the effects of the latter. Pellets composed of cholesterol, glyceryl stearate and naltrexone implanted s.c. in rats were found to confer blockade of the analgesic effects of morphine for up to 2 months, offering an improved long-term delivery system for antagonists.[86] A 6 β-N mustard analog of naltrexone, 9, covalently binds to the opioid receptor, conferring narcotic antagonist

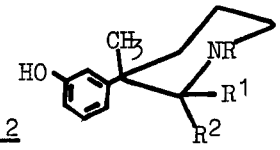
activity, detectable up to 3 days, without agonist effects. 3-Methoxy-8-oxamorphinans, 10, show pharmacological properties essentially similar to 9-OH benzomorphans.[83]



10



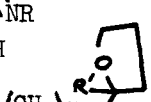
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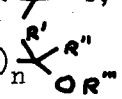


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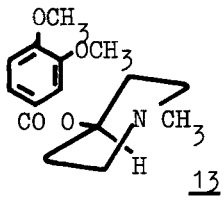
a, $R=CH_2\text{-}c\text{-}C_3H_5$, $R^1=H$, $R^2=CH_3$

b, $R = CH_2\text{-}c\text{-}C_3H_5$, $R^1=CH_3$, $R^2=H$

R^1 = OH a) $R = (CH_2)_n$ 

$R^2 = CH_3$ b) $R = (CH_2)_n$ 

c) $R^1 = R^2$=H, R=CH_3



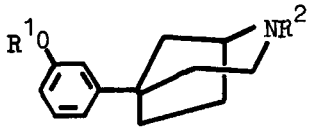
13

Benzomorphans - The activities of N-alkoxyalkylnormetazocines, 11 a-b, show large variations,[89] and the SAR suggests interactions of high stereospecificity for the receptor environment around the N-substituents. The activity of NIH 8933, 11c, is shown not to be due to an impurity or to metabolic hydroxylation in vitro, indicating that an oxygen atom is not essential for interaction of an agonist with the opioid receptor.[90] Interesting previously-reported 3-benzazocines possessing nitro groups have been shown to be long-acting antagonists, partially antagonizing up to about 50% of the antinociceptive effect of 20 mg/kg of morphine, for over 48 hrs.[91]

Substituted Piperidines - The pharmacokinetics of fentanyl have been determined by using radioimmunoassay.[92,93] In man, at effective analgetic doses, the action of fentanyl may be sufficiently prolonged to cause a delayed respiratory depression, after apparent recovery from anesthesia.[94] Phenobarbitone has been shown to enhance the demethylation of meperidine, to the toxic normeperidine, by enzyme induction.[95] Some 2,3-dimethyl-3-arylpiperidines, 12, with allyl, CH_2-c-C_3H_5, and dimethylallyl N-substituents show a range of antagonist potencies. The β-2-methyl derivatives showed antagonism of the effects (respiration, righting reflex, rigidity and analgesia) of fentanyl (0.63 mg/kg, s.c.), with a potency similar to that of nalorphine. Substitution of a para hydroxyl for the meta hydroxyl led to a fall in antagonist activity. Isomers 12a and 12b acted in the single dose suppression test as potent pure antagonists. Aromatic esters of 1-methyl-4-piperidinol have a wide range of activity in the range 0.2-1 x codeine (hot plate).[97] One derivative, 13 (0.33 x codeine), is reported to be free of morphine-like physical dependence liability in monkeys.

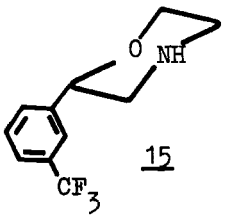
Miscellaneous - Several 5-aryl-2-azabicyclo (3.2.1.) octanes, 14, are partial agonists with potencies comparable to morphine, the phenethyl derivatives being the most potent.[98] Antagonist pharmacophores (CH_2-c-C_3H_5, dimethylallyl) resulted in little increase in agonist or antagonist potency, while the furylmethyl derivative was a good antagonist without much increase in analgesic potency. The derivative, 14a, at 50 and 100 mg/kg/day, fails to substitute for morphine in rats infused with morphine at 50, 100, 4 x 200 mg/kg/day.

An aryloxazine derivative, 15, is reported to be more potent than morphine in the hot plate and phenylquinone writhing tests, and about equipotent with morphine in blocking acetic acid writhing. It did not lead to tolerance or dependence in rats.[99] Two examples, 16a, and 16b, from a series of 1,2,3,4,5,6-hexahydro-1,6-methano-3-benzazocines have shown potencies greater than codeine in the hot plate test, while a third example, 16c, was somewhat less potent.[100] Antagonist pharmacophores (CH_2-c-C_3H_5,C_3H_7, allyl, dimethylallyl) did not give antagonists. It is reported that 16a and 16b will neither support morphine dependence in single dose suppression studies, nor precipitate withdrawal symptoms in morphine addicted non-withdrawn rhesus monkeys. Spiro (tetralin-2,2'-pyrrolidine) and spiro (indan-2,2'-pyrrolidine) derivatives of structure 17 showed some activity.[101] The best derivatives, R^1=H, R^2=H, Me, n=2, were less potent than morphine. Some long-acting piperidinospiro derivatives of methadone have potencies up to 212 x methadone and durations of action of up to 20.5 hours.[102]

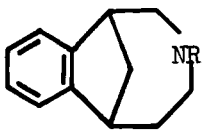


14

a, R^1 =H,R^2 = $PhCH_2CH_2$

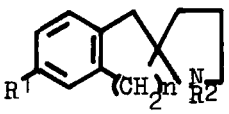


15



16

a, R = H
b, R = CH_3
c, R = CH_2CH_2Ph



17

n = 1,2
R^1 = H, OCH_3,OH
R^2 = H, CH_3

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Chapter 5. Amino Acid Neurotransmitter Candidates

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Biochemical, electrophysiological and pharmacological studies indicate that certain amino acids may serve as neurotransmitters or neuromodulators in the mammalian central nervous system (CNS). In spite of the impressive amount of research conducted on this subject, the application of this knowledge to the treatment of CNS disorders has been limited due to the paucity of chemical agents which will, after systemic administration, act in a specific fashion on these neurotransmitter systems. A major reason for the delay in the development of effective therapeutic agents is that, of the sixteen electrophysiologically active amino acids present in the mammalian CNS, there are only two, γ-aminobutyric acid (GABA) and glycine, for which potent antagonists are known. Without potent, relatively specific antagonists it is virtually impossible to fully characterize the structure-activity requirements of a neurotransmitter system since specificity of action cannot be demonstrated. Thus, much work remains with respect to the chemistry of these transmitter systems.

The present communication is intended as a brief overview of the literature pertaining to amino acid transmitter candidates. Interested readers are urged to consult any of a number of excellent reviews.[1-7]

Neurotransmitter candidates may be classified as inhibitory or excitatory, depending upon the electrophysiological response to the agent. Inhibitory transmitters cause hyperpolarization or partial depolarization of nerve cells, inhibiting cell firing, whereas excitatory transmitters cause depolarization sufficient to generate an action potential. With regard to amino acid transmitter candidates, twelve substances have been identified in the mammalian central nervous system which, based on electrophysiological data, may be inhibitory, and four which appear to be excitatory[3] (Table 1). However, before a substance can be seriously considered as a neurotransmitter candidate, several criteria, relating to the transport, storage, release and termination of action must be fulfilled. Of the agents listed in Table 1, five--taurine, glycine, GABA, glutamic acid and aspartic acid--have been studied sufficiently to indicate the likelihood of a neurotransmitter action. These five will be considered in more detail in the following paragraphs.

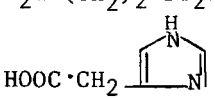
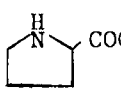
Inhibitory Amino Acids

Inhibitory amino acids are sometimes subdivided into GABA-like and glycine-like since, at present, the action of only these two amino acids can be pharmacologically differentiated from one another. Thus, strychnine (17) antagonizes glycine but not GABA, and bicuculline (18) antagonized GABA but not glycine. However, the action of a number of other amino acids is blocked by strychnine or bicuculline, while the action of some, on certain cells at least, is blocked by both agents. Accordingly, bicuculline and strychnine are referred to as GABA and glycine antagonists, respectively, because these two amino acids are the most sensitive

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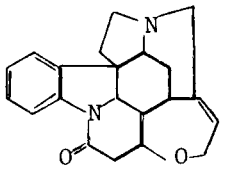
TABLE 1

ELECTROPHYSIOLOGICALLY ACTIVE AMINO ACIDS PRESENT IN MAMMALIAN CENTRAL NERVOUS SYSTEM

Amino Acid	Structure	Reference
Inhibitory		
L-α-Alanine	$CH_3 \cdot CH(NH_2) \cdot COOH$ 1	8
β-Alanine	$H_2N \cdot (CH_2)_2 \cdot COOH$ 2	9
γ-Aminobutyric Acid (GABA)	$H_2N \cdot (CH_2)_3 \cdot COOH$ 3	10,11
γ-Amino-β-hydroxybutyric Acid	$H_2N \cdot CH_2 \cdot CH(OH) \cdot CH_2 \cdot COOH$ 4	12
L-Cystathionine	$HOOC \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$ 5	13
2,4-Diaminobutyric Acid (DABA)	$H_2N \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$ 6	14
Glycine	$H_2N \cdot CH_2 \cdot COOH$ 7	10,15,16
Hypotaurine	$H_2N \cdot (CH_2)_2 \cdot SO_2H$ 8	5
Imidazole-4-acetic Acid	 9	12,17
Proline	 10	3
L-Serine	$HO \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ 11	8
Taurine	$H_2N \cdot (CH_2)_2 \cdot SO_3H$ 12	18
Excitatory		
L-Aspartic Acid	$HOOC \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ 13	19
L-Cysteic Acid	$HO_3S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ 14	19
L-Cysteine Sulphinic Acid	$HO_2S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ 15	20
L-Glutamic Acid	$HOOC \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$ 16	19,21

to the action of these alkaloids.

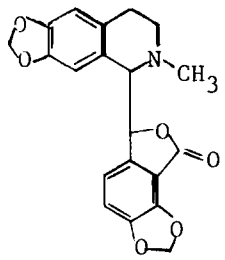
Taurine - A glycine-like, sulphur containing amino acid, taurine (12) is found in reasonably high concentrations throughout the mammalian central nervous system and in heart.[22,23] In brain, taurine is formed as a result of the decarboxylation of cysteine sulphinic acid (15) to hypotaurine (8), which in turn is oxidized to form taurine.[24] Like other transmitter candidates, taurine is accumulated and released by brain tissue and the accumulation can be inhibited by ouabain. Clinically, significant alterations in taurine levels may be associated with retinitis pigmentosa,[27] epilepsy,[28] mongolism,[29] and possibly heart disease.[30] Reports have suggested that systemic administration of taurine may be efficacious in treating epilepsy.[31,32] Like glycine, the action of taurine is inhibited by strychnine,[8] though in other brain areas, such as the cerebral cortex, its action is blocked by both strychnine and bicuculline.[9] Thus, unlike glycine and GABA, there is no antagonist for taurine which will differentiate taurine receptors from other inhibitory amino acid receptor sites.



17

$H_2N \cdot (CH_2)_3 \cdot SO_3H$ $HO_3S \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$

19 20

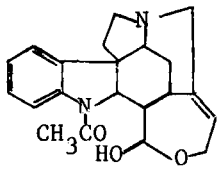


18

In this regard, it is interesting to note that another sulphur containing amino acid related to taurine, 3-amino-1-propanesulfonic acid (19), is a potent, and relatively specific, (bicuculline-sensitive, strychnine-insensitive) GABA receptor agonist,[33,34] which further suggests a lack of specificity for this class of compounds. Other derivatives such as N-methyltaurine, N-carbamoyltaurine, 4-aminobutanesulfonic acid and the taurine metabolite isethionic acid are very weak or ineffective when applied to cortical neurones or spinal cord.[33,35,36] The structure-activity relationship of the taurine receptor is further complicated by the fact that two other structurally related amino acids, L-cysteic acid (14) and DL-homocysteic acid (20), are potent excitatory agents.[33] Thus, further pharmacological studies to characterize the putative neurotransmitter receptor site for taurine must await the discovery of compounds capable of specifically inhibiting the action of this substance.

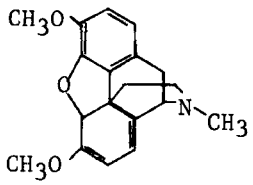
Glycine - The simplest amino acid, glycine (7) has a unique distribution within the mammalian central nervous system, being highest in the medulla and spinal cord.[37] This finding, along with autoradiographic and electrophysiological studies, have suggested that glycine may serve as a neurotransmitter primarily in the spinal cord and brain stem.[8,38] There are three major pathways for glycine synthesis; glycine from glucose via serine (11); glycine by way of a phosphorylated pathway;[39] glycine by way of a dephosphorylated pathway.[40] Within the central nervous system glycine does not appear to be catabolized as such, but rather the majority

is incorporated into proteins and nucleotides. Glycine is accumulated by a high affinity transport system into spinal cord, pons and medulla,[41,42] and has been shown to be released from nervous tissue under appropriate conditions.[43] The majority of experimental evidence suggests that, in the spinal cord, glycine is the predominating inhibitory neurotransmitter at synapses on motor neurones, spinal interneurones, in dorsal column nuclei and medullary reticular formation.[44] Also, glycine may serve as a transmitter in retina.[45] However, as yet, there is little evidence directly linking neurotransmitter glycine to any specific neurological or psychiatric disorder, though a glycine abnormality may be associated with spasticity.[46]



21

Intracellular recording studies with glycine indicate that it hyperpolarizes neurones and increases membrane conductance.[13] A number of compounds block the action of glycine, with strychnine being the most potent. Other glycine antagonists include diaboline (21), thebaine (22), gelsemine, dendrobine, brucine and 4-phenyl-4-formyl-N-methylpiperidine.[3] While these agents are useful for differentiating glycine from GABA receptors, they will also antagonize the inhibition induced by other amino acids such as serine (11), cystathionine (5), taurine and α and β-alanine (2).[8]



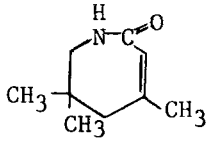
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Studies have shown that, when incubated under the proper conditions, ^{3}H-strychnine binds in a selective fashion to spinal cord membranes and this binding appears to be associated with glycine receptors.[47] The potency of various amino acids to inhibit ^{3}H-strychnine binding parallels their electrophysiological potencies on strychnine sensitive receptors, with glycine and β-alanine being the most potent. Also, binding studies with strychnine have indicated that the receptor for the alkaloid may be more related to the ion conductance mechanism of the glycine receptor than to the receptor recognition site to which glycine attaches.[48] If a single ion channel can be activated by a number of different receptor agonists acting at different recognition sites, this finding may explain why strychnine is capable of reversing the action of a number of amino acids. On the other hand, it is also possible that agents such as β-alanine, proline (10) and taurine, while perhaps not neurotransmitters themselves, have some affinity for the glycine recognition site. The latter explanation would seem most likely if the antagonism of glycine by strychnine is a competitive phenomenon, as has been suggested by some workers.[49]

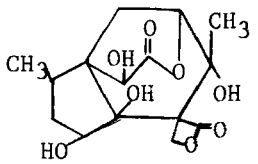
At present there are no specific glycine receptor agonists more potent than glycine itself, and there are no agents which can specifically alter the neuronal metabolism, uptake or release of this amino acid making it difficult to selectively manipulate this system.

GABA - Of all the amino acid neurotransmitter candidates, GABA (3) has been the most comprehensively studied. Numerous reviews have been published concerning the biochemical, physiological and pharmacological actions of GABA, one of the most recent of which appeared in Volume 13 of this series.[4-6,50-53] Thus GABA will be discussed only in terms of the most recent findings.

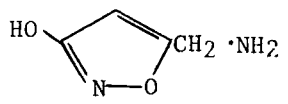
Electrophysiological and biochemical studies have provided considerable evidence to suggest that GABA is an inhibitory neurotransmitter in the mammalian central nervous system. In addition, studies have indicated that alterations in GABAergic transmission may underlie the symptoms of numerous neurological and psychiatric disorders.[54-57] Because of these findings, and because the metabolic pathways for the synthesis and degradation of GABA are well defined, numerous compounds have been synthesized which can affect, quite selectively in some cases, GABA synthesis, degradation, transport and postsynaptic receptor activity.[50-51] Most of the research in this area has been directed towards finding agents which will enhance GABAergic transmission since agents which decrease GABA function, such as the receptor blocker bicuculline, are convulsants. Other GABA receptor blocking agents include picrotoxin,[50] tetramethylenedisulphotetramine[58,59] and some bicyclophosphate esters.[60,61] In addition, the GABA receptor appears to be blocked by certain caprolactams,[62] such as 4,6,6-trimethyl-Δ3-caprolactam (23), and by the alkaloid shikimin (24).[63]



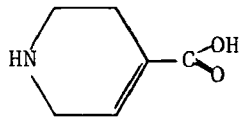
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With regard to direct acting GABA receptor agonists, much work has been devoted over the past few years to synthesizing and studying isoxazole derivatives related to muscimol (25) and to cyclic amino carboxylic acids, such as isoguvacine (26).[64] The reason for the interest in these structures is that muscimol appears to be significantly more potent than GABA as a GABA receptor agonist.[65,66] As a result of these studies several new, relatively potent and specific, GABA agonists have been synthesized. However, while these compounds have been invaluable in understanding the pharmacological specificity of the GABA system, there is some question as to whether they will be useful therapeutic agents, because of their low lipid solubility and rapid metabolism after systemic administration.[67]

Recent experiments on the physicochemical properties of the GABA receptor have revealed that there is present on the nerve cell membrane a triton-sensitive substance which interferes with the attachment of GABA to its receptor recognition site.[68,69] Careful biochemical studies have suggested that this triton-sensitive substance modifies the affinity of the GABA receptor site in a non-competitive manner, suggesting that this substance, or modulator, interacts with a membrane component to produce an allosteric change

in the conformation of the receptor site.[70] Further experiments indicate that benzodiazepines, like triton, can remove this inhibitory substance, with resultant enhanced attraction of the receptor for GABA. Thus, these findings suggest the possibility that the mechanism of action of the benzodiazepines may be to facilitate GABAergic transmission by removing an endogenous modulator of GABA binding from the vicinity of the recognition site. These biochemical findings support earlier electrophysiological results indicating that benzodiazepines enhance GABAergic transmission.[71-73]

Thus significant progress has been made with respect to the pharmacology of the GABA system. However, it should be pointed out that electrophysiological and biochemical studies have shown that other endogenous amino acids, such as γ-amino-β-hydroxybutyric acid (4), 2,4-diaminobutyric acid (6) and imidazole-4-acetic acid (9), can also interact with the GABA receptor and transport sites[12,74] though they are less potent than GABA. Clarification as to whether these substances, which are present in only minute concentrations in the CNS, are neurotransmitters in their own right must await the development of agents which will specifically antagonize their actions.

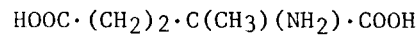
Excitatory Amino Acids

Glutamic and Aspartic Acids - These two amino acids will be considered together since they have a similar action and distribution in the central nervous system and since there is as yet no antagonist which can totally differentiate the action of these agents.

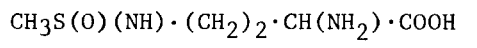
As opposed to the two other endogenous excitatory amino acid candidates, cysteic acid and cysteine sulphinic acid (15), glutamate (16) and aspartate (13) are found in abundant quantities in the mammalian CNS.[75,76] Metabolically, aspartate and glutamate are related and their metabolism is quite complex. Thus, there are undoubtedly several metabolic pools of glutamate and aspartate, in addition to any neurotransmitter pool, making it difficult to study the biochemical aspects of their neurotransmitter action. Nevertheless, it has been shown that both substances are accumulated into brain tissue by a high affinity process,[77] and that both can be released from brain tissue by electrical field stimulation.[78] This behavior is characteristic of neurotransmitters.

While the regional distribution is fairly uniform for these two substances, there may be significant differences in the concentrations of aspartate and glutamate in certain areas of the spinal cord.[79] Furthermore, biochemical studies have suggested that aspartate may be concentrated in the interneurons of the polysynaptic reflex arc,[80] and that there may be a specific glutamate pathway from the cortex to the corpus striatum.[81,82]

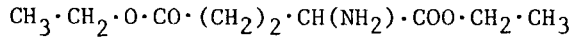
With respect to their postsynaptic action, both glutamate and aspartate evoke an excitatory response from central nervous system neurons.[19,21] However, while there are a number of agents which will inhibit the excitatory action of these substances, none will completely differentiate between the two. Thus, glutamate-aspartate antagonists incluce DL-α-



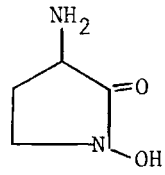
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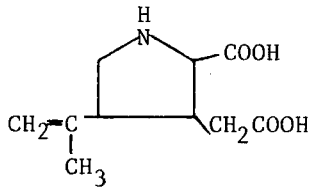


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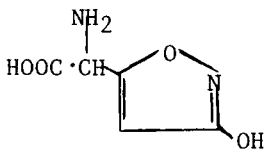
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methylglutamic acid (27), L-methionine-DL-sulfoximine (28), L-glutamic acid diethyl ester (29), and 1-hydroxy-3-amino pyrrolidone-2 (30).[3] Of these compounds only one, the diethyl ester (29), may be significantly more potent as an inhibitor of glutamate than aspartate,[83,84] whereas the others are essentially equipotent, making it difficult to differnetiate the action of the two amino acids.



31

Based on structural and electrophysiological similarities, a number of compounds have been proposed as glutamate agonists. Some of the most potent of these substances are kainic acid (31), ibotenic acid (32), cyclopentane glutamate (33) and quisqualic acid (34).[85,86] It is noteworthy that electrophysiological experiments suggest that kainic acid, as opposed to the other compounds, may not activate glutamate receptors directly, but rather may, in some way, allosterically sensitize the receptor to the action of endogenous glutamate.[87,88] While it has been suggested that these compounds are specific agonists for glutamate vis-s-vis aspartate, such specificity cannot be absolutely proven until agents are available which clearly differentiate the excitatory action of glutamate from aspartate.

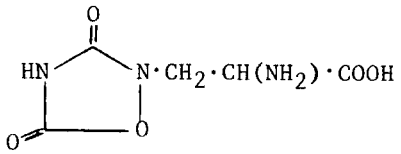


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It has recently been discovered that some of the excitant amino acids and their analogues, such as kainic acid, are nerve cell toxins. That is, when administered to immature animals, either glutamate or aspartate causes degeneration of retinal and hypothalamic cells[89] and, when injected into brain, kainic acid causes irreversible destruction of virtually all neuronal cell bodies around the injection site.[90,91] These discoveries may be of immense importance with respect to understanding the etiology of some neurodegenerative disorders since they suggest that an overabundance of excitatory amino acids in the brain may cause progressive cell loss. Therefore,



34

potent, and specific antagonists for excitant amino acids may be capable of preventing, or delaying, some types of neuronal degeneration.

Conclusions

Substantial evidence has been accumulated suggesting that certain amino acids may serve as neurotransmitters in brain. However, little is yet known about the pharmacology of these systems. One reason for the delayed progress in this area is that, as opposed to other neurotransmitters such as the catecholamines, the transmitter function of amino acids is only one of perhaps several actions of these agents making it difficult to identify the neurotransmitter pool. The structural and electrophysiological similarities of the amino acids are other hindrances to the study of these compounds. Nevertheless, the possibility that some or all may be neurotransmitters is great enough to warrant continued study since there is evidence suggesting that the pharmacological manipulation of amino acid transmitter systems may be beneficial in a variety of CNS disorders. What makes them especially tempting in this regard is the fact that amino acids appear to act as transmitters only in the CNS. Therefore, drugs which will activate or depress these transmitter systems may have little or no direct action on peripheral organs making them somewhat more specific, and therefore less toxic, than other neuropharmacological agents.

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Section II - PHARMACODYNAMIC AGENTS

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Chapter 6. Pulmonary and Anti-Allergy Drugs

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Introduction - The immunopathologic events involved in an asthmatic attack can be generally classified as a Type I immediate hypersensitivity reaction involving specific antigen bridging receptive IgE immunoglobulins bound to host mast cells and basophils that culminates in the release of various pharmacologic mediators.[1] The mechanisms responsible for the release of these mediators have been extensively reviewed.[1-3]

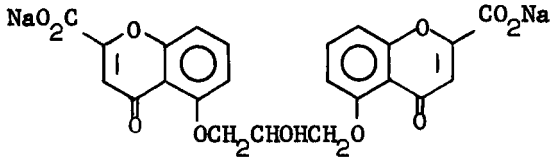
Traditionally, drugs used in the treatment of asthma can be conveniently categorized into 4 classes: methylxanthines, β-adrenergic stimulants, glucocorticoids and anticholinergics.[4] In recent years, however, the clinical efficacy of cromolyn sodium as a prophylactic anti-allergy agent has stimulated a substantial research effort to find more potent, orally active inhibitors of mediator release. One of the primary assay systems designed to evaluate potential antiallergic compounds of this type is the passive cutaneous anaphylaxis (PCA) test in rats. The fact that compounds in the PCA test, e.g., bufrolin and doxantrazole have been less than promising in clinical trials of asthma, has shed doubt on the capacity of this test to predict clinical efficacy. Longer treatment times may be necessary to achieve clinical benefit with this class of compounds or perhaps there are as yet ill-defined pathologic mechanisms involved in asthma that are unaccounted for in experimental models.

Given the immunopathologic events involved in allergic diseases and the multitude of mediators released from triggered sensitized cells, future trends for exploration of drug action designed to interrupt or regulate this sequence of events should include: 1) compounds with the capacity to inhibit mediator release, as well as antagonize specific mediators through receptor blockade, i.e., compounds exhibiting polypharmacology,[5] 2) receptor antagonists with broncho specificity,[6] 3) the development of specific antagonists of IgE binding to mast cells and/or basophils[7] and 4) the development of compounds with immunoregulatory activity capable of influencing the synthesis and/or secretion of IgE molecules.[8] Although a formidable task, development of compounds of this nature should lead to rational therapy for allergic diseases.

Inhibitors of Mediator Release - Several reviews have examined the role of mediators in the pathophysiology of asthma and other allergic disorders.[9] Numerous pharmacologic agents have been introduced which inhibit the mechanisms responsible for the release of these mediators.[10-12] Their actions and clinical efficacy have been compared

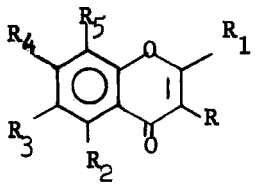
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to the prototype drug of this class - disodium cromoglycate (DSCG) 1.



1

Analogs of 1 FPL-52757 (2) and FPL-57787 (3) have been reported[11] to be orally effective in clinical trials while 4 was ineffective in asthmatics.[13] A tetrazole analog AA-344 (5) was 4 times as active as DSCG in the rat PCA and orally effective in 7 of 14 asthmatic patients.[14,15] Of these 7 patients, 6 were RAST positive with high serum IgE levels.[16] Although the tetrazolecarbonyl derivative 6 was very potent in the rat PCA test by the i.v. route (ED_{50}=.037 mg/kg), it was much less potent by oral administration (ED_{50}=21 mg/kg).[17] Clinical investigation reveals comparable activity to DSCG.[17] Benzopyrones 7 containing a carboxy group in the 6-position and a 2-pyridyl group in the 2-position had comparable i.p. potency to DSCG in the rat PCA test and were orally active at doses of 5-10 mg/kg.[18]



2, R_1=CO_2H, R_2=OH, R_3, R_4=Et

3, R_1=CO_2H, R_2=OH, R_3, R_4=$(CH_2)_4$, R_5=C_3H_7

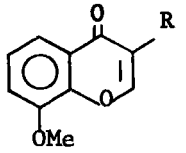
4, R_1=CO_2H, R_2=$CH_2CHOHMe$

5, R=tetrazole, R_3=Et

6, R_1=CONH-tetrazole, R_4=OMe

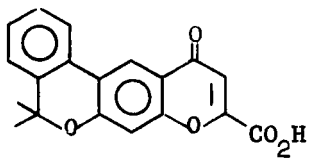
7, R_1=2-pyridyl, R_3=CO_2H

W-8011 (8) a 3-hydroxymethyl benzopyrone was active in the rat PCA (ID_{50}=2 mg/kg p.o.) but inactive in vitro, suggesting that the active form was 3-carboxy-8-methoxychromone 9.[19]

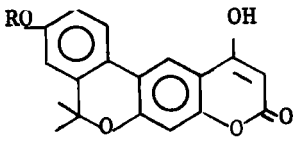


8, R=CH_2OH

9, R=CO_2H



10



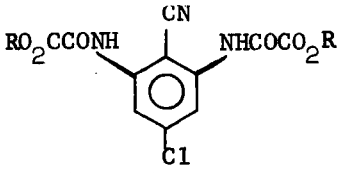
11, R=Me

12, R=CH_2CH_2OH

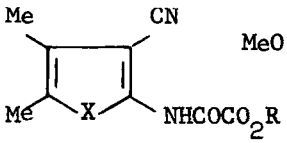
The tetracyclic pyrone PRD-92-EA (10) had an i.v. ED_{50} of 0.5 mg/kg in the rat PCA and was only slightly effective in the IgG mediated PCA in the guinea pig.[20] Compound 10 also inhibits the effects of mediators, i.e., histamine, 5-HT, bradykinin, SRS-A on guinea pig ileum[20] and on cardiovascular responses in fowl.[21] Compounds 11 and 12, related in

structure to 10, were reported to be not only inhibitors of mediator release but also antagonists of pharmacologic mediators of anaphylaxis.[22]

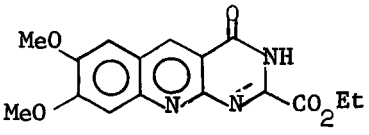
Lodoxamide tromethamine (U-42585E, 13) is 2500 X more potent than DSCG in the rat PCA test[23] administered i.v., and is orally active in ascaris sensitive monkeys at 5 mg/kg.[24] In clinical studies, 13 appears to protect extrinsic asthmatics from allergen induced bronchoconstriction administered by the aerosol route, and 5 of 10 asthmatic patients were protected by lodoxamide given orally at doses of 1 and 2 mg.[25]



13, R=$NH_3C(CH_2OH)_3$
14, R=Et



15, X=NH or O

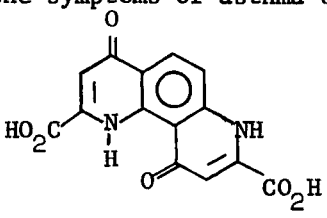


16

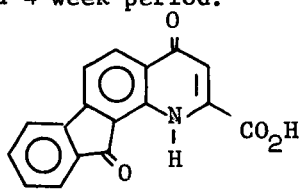
The ethyl ester (14) is reported to be similar in potency to the tromethamine salt (13).[26] Clinical testing of 14 has shown it to effectively block antigen bronchoprovocation in asthmatic subjects at oral doses of 1 and 3 mg;[27,28] however, higher oral doses (10 mg) produced intolerable side effects such as: weakness, nausea, diaphoresis, a feeling of warmth, and in 2 cases, vomiting and diarrhea. Furan and pyrrole o-cyanooxamates 15[29] were less potent than 13.

A series of 2-carbethoxypyrimidoquinolines, exemplified by compound 16, had potent oral activity[30a] and were up to 400 X as potent as DSCG by the i.v. route in the PCA test.[30b]

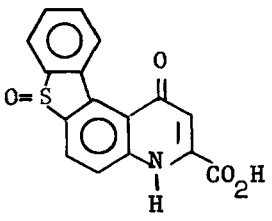
Bufrolin, ICI74917 (17), although effective in allergen induced bronchoconstriction in asthmatic patients, failed to clinically improve the symptoms of asthma over a 4 week period.[11,17]



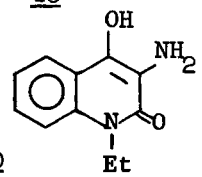
17



18



19

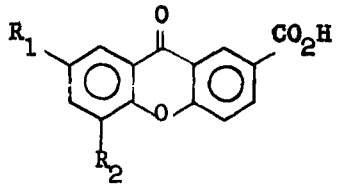


20

The tetracyclic pyridone-2-carboxylic acid (18), similar in structure to 17, was equipotent[32] to DSCG while a sulfonyl analog (19) was up to 8 X more potent than DSCG in the rat PCA model.[33] A series of 3-aminoquinolones (ex. 20) proved to be less effective than DSCG in the rat PCA test.[34]

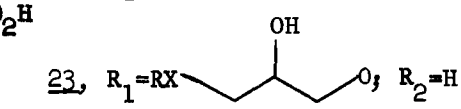
Using molecular orbital theory, a significant correlation was found between the LUMO and the biological activity in a series of oxamates, quinaldic, and benzopyran-2-carboxylic acids.[35] This was rationalized in terms of charge transfer stabilization of the drug-receptor complex.

Numerous xanthone derivatives have undergone clinical investigation. AH-7725 (21) was comparable to DSCG in clinical trials,[11] while xanoxic acid (22) was reported to be effective in exercise-induced asthma.



21, R_1=$HOCH_2CH_2O$; R_2=H

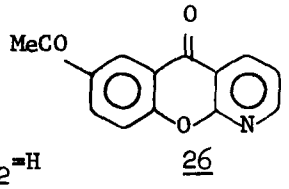
22, R_1=Me_2CHO; R_2=H



23, R_1=RX O; R_2=H

24, R_1=MeCO; R_2=H

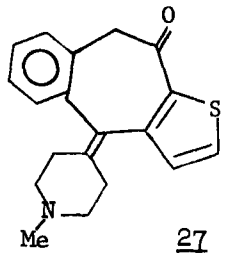
25, R_1=MeS(=O)NH; R_2=hexyl



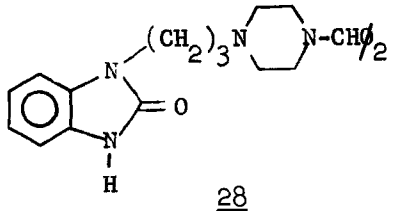
26

Derivatives of 21, i.e. 23, were active by the i.p. route in the rat PCA test but inactive orally.[36] The 6-acetyl analog 24 was reported to be 6 X as potent as DSCG in the rat PCA test[37] and a 6-sulfonimide,[38] RU31156 (25) was 263 X more potent than DSCG by the i.v. route in the PCA test. The ring nitrogen analog Y-9000 (26) was similar in activity to DSCG and in addition was orally active.[39,40]

Several clinical trials have shown ketotifen HC-20-511 (27) to be an effective antiasthmatic compound and in fact the compound has been marketed in several European countries.[41-47] In addition to having anti-anaphylactic properties, 27 is also a potent long acting H_1 receptor antagonist.[48] Ketotifen has also been shown to inhibit c-AMP phosphodiesterase. Oxatomide (28) presumably inhibits mediator release from mast cells[49] and demonstrates non-competitive antihistaminic activity.[50,51] In a large clinical trial, 28 was effective in allergic rhinitis.[51]

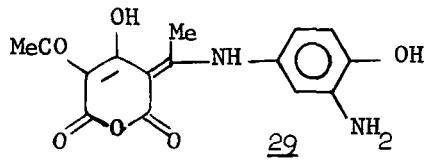


27

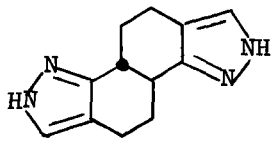


28

A series of antiallergic pyranenamines was developed with the aid of QSAR.[52] The clinical candidate of this class of compound appears to be SKF-78929-A (29) which has an ID_{50} of 0.7 mg/kg by both the i.v. and oral routes. Compound 29 also produced a dose dependent inhibition of immunologic release of histamine and SRS-A from passively sensitized rhesus monkey and canine lung.[53]



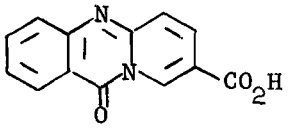
29



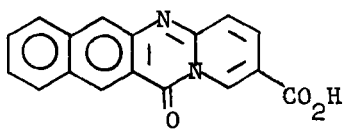
30

The tetracyclic pyrazole 30 (trans form) has an oral ED_{50} of 35 mg/kg in the rat PCA.[54,55] In contrast to DSCG, 30 exhibited activity for 6 hours when doses 4 X higher than the ED_{50} were administered. Predose of the compound increased its effectiveness.

A series of quinazolinecarboxylic acids (31, 32) have been synthesized and evaluated as potential antiallergic agents.[56] Compound 31 was 10-20 X more potent than DSCG or doxantrazole intravenously, but inactive orally in the PCA test.[56] Compound 32 was 5-10 X more potent than DSCG and also was orally inactive. Synthesis and biologic evaluation of analogs of 32 are in progress.[56]

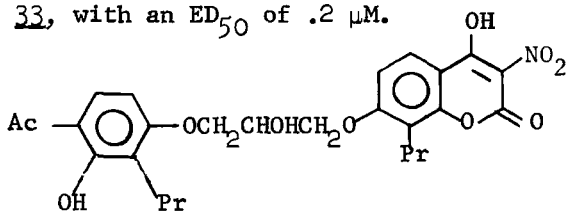


31



32

A series of 4-hydroxy-3-nitrocoumarins had potent activity in the PPA (passive peritoneal anaphylaxis) test (about 10 X DSCG). These compounds also antagonized the effects of SRS-A[57] and one of the most potent analogs was 33, with an ED_{50} of .2 μM.

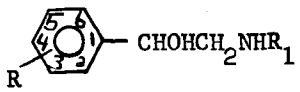


33

β-Adrenergic Stimulants - The activity, potency, and selectivity of a number of β-adrenergic agonists has been reviewed.[58] Sympathomimetic bronchodilator drugs are of considerable value in the treatment of some obstructive airway diseases but side effects, particularly on the heart, have prompted the search for new derivatives in recent years with higher β_2/β_1 selectivity. With the advent of newer agents with high β_2 activity, muscle tremor, which is β_2 mediated, appears to be the most common side effect of long term maintenance therapy.[59-61]

The pharmacology and clinical effectiveness of fenoterol (34) has been recently reviewed[62] with comparisons made to isoproterenol (35), orciprenaline (36) and salbutamol (37). Clinical experience has shown that fenoterol, a highly selective β_2 agonist, is an effective bronchodilator with negligable effects on the cardiovascular system following

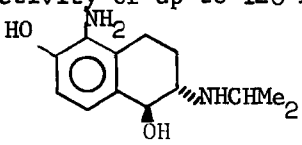
aerosol administration of usual therapeutic doses.[62]



34, R=3,5-di-OH, R_1=$CHMeCH_2C_6H_4$-p-OH
35, R=3,4-di-OH, R_1=isopropyl
36, R=3,5-di-OH, R_1=isopropyl
37, R=4-OH-3-CH_2OH, R_1=t-butyl
38, R=3-OH-4-$NHSO_2Me$, R_1=$CMe_2CH_2C_6H_5$
39, R=4-Cl, R_1=t-butyl

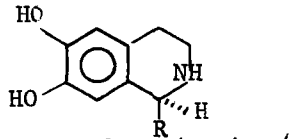
Zinterol (MJ9184, 38), which is structurally similar to 34, is a long acting bronchodilator, less potent than isoproterenol, and not highly selective for β_2 receptors. The agent is orally effective at doses as low as 0.25 mg in asthmatic patients but significant increase in pulse rate at somewhat higher doses of .5 mg were observed.[64] A p-chloro analog (39) was reported to have a 20 X greater β_2/β_1 selectivity than isoproterenol.[65]

Several papers have described the synthesis and biologic activity of cyclic phenethanolamines (i.e. tetrahydronaphthalenes, ex. 40),[66-68] some of which have β_2 selectivity of up to 120 X that of isoproterenol.

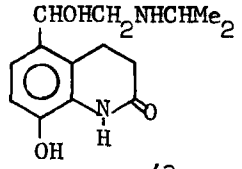


40

The synthesis and pharmacologic activity of erythro and threoisomers of trimethoquinol (42) have been studied and the α-hydroxy erythro isomer 41 was shown to be a more potent adrenoceptor stimulant than the threo isomer but less potent than 42.[69]



41, R=-CHOH-2,3,4(OMe)$_3$-Ø
42, R=-CH_2-2,3,4(OMe)$_3$-Ø



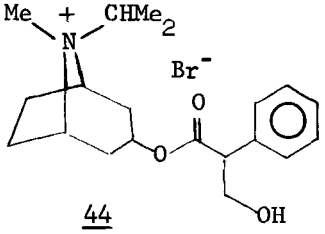
43

A quinolone ethanolamine derivative 43 is one of the most selective β_2 adrenergic receptor stimulants described to date.[70,71] The compound, like salbutamol, is a partial agonist on β_1 cardiac adrenergic receptors.[70]

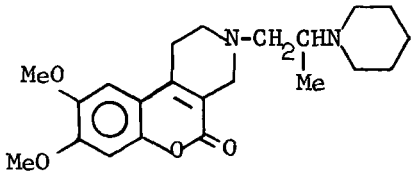
Anticholinergics - There is increasing evidence suggesting a role for cholinergic mediated reflex bronchoconstriction as an etiologic factor in obstructive lung disease.[72,73]

Ipratropium bromide (Sch 1000, 44) continues to be of clinical interest. The compound has been shown to be superior to fenoterol at doses of 40 μg as an aerosol. Effectiveness is diminished at higher

doses, possibly due to impairment of mucus transport.[74] C1-864 (45) is a broncho-selective antimuscarinic agent which is orally active in animals and compared to atropine has reduced effects on the CNS, heart, secretory mechanisms, and pupil diameter.[6]

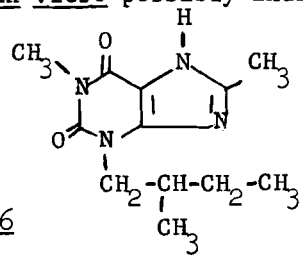


44

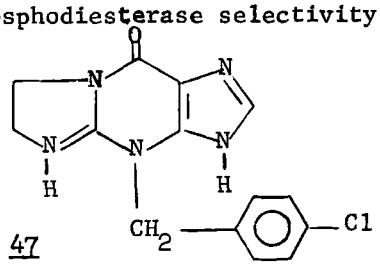


45

Phosphodiesterase Inhibitor - CK-0383 (46) was 170 X more potent and 5 X more selective than theophylline tested in guinea pig trachea and atrium in vitro possibly indicating phosphodiesterase selectivity.[75]



46



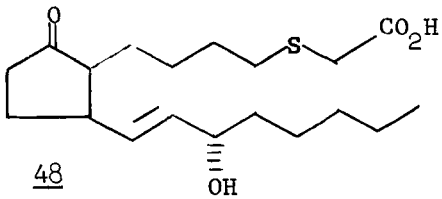
47

7-Acyl derivatives of theophylline were prepared as prodrugs.[76] The compound of choice was 7,7'-succinylditheophylline.

A tricyclic heterocycle (M.J. 12504, 47) described as having polypharmacology, is reported to be more potent than theophylline in the rat and to have less CNS and cardiovascular side effects. The compound also inhibits mediator release.[77]

Prostaglandins - Several reports indicate that the products of arachidonic acid metabolism, i.e., prostaglandins, prostacyclins and thromboxanes are involved in the pathophysiology of obstructive lung disease.[78-80] Prostaglandins of the E series are bronchodilators while prostaglandins of the F series are bronchoconstrictors.

Synthetic prostaglandins similar to PGE continue to be of potential interest in asthma therapy. The sulfur containing analog 48 was reported to be an active bronchodilator but much less potent than PGE_1.[81]



48

Recent studies have suggested that prostacyclin (PGI_2) and thromboxane (TxA_2) inhibit the release of histamine and SRS-A from lung tissue during anaphylaxis.[82] These findings may indicate a modulating role in asthma for these compounds.

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Chapter 7. Antihypertensive Agents

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Introduction - The search for new antihypertensives and for a better understanding of existing ones remains quite intense with emphasis this past year on β-blockers (see Chapter 9) and on agents that affect the renin-angiotensin system. Diuretics were reviewed last year and are not included in this chapter. Structures of drugs mentioned in this review are shown or may be found in recent volumes of Annual Reports in Medicinal Chemistry.

General - Clinical papers and reviews[1-6] reflect a definite adoption by practitioners of the "stepped-care" approach to drug selection mentioned last year. This empirical approach to therapy does not identify the underlying pathophysiology in individual patients but is useful for controlling the blood pressure of most patients. In contrast, some clinicians advocate profiling of patients by measurement of hemodynamic and hormonal parameters; therapy is then prescribed according to the mechanism responsible for the elevated blood pressure. "Renin-profiling" identifies patients with renin-dependent and those with volume-dependent hypertension;[7] the former are then treated first with β-blockers or inhibitors of the renin-angiotensin system, and the latter with diuretics. However, this approach is not universally accepted or successful.[8] Measurement of sympathetic nervous activity by recently developed, highly sensitive assays for norepinephrine (NE) levels may help determine the etiology and basis for therapy in the 30-40% of essential hypertensives who are "hyperadrenergic."[9,10] A particularly good correlation has been found between blood pressure and a combination of plasma NE levels and reactivity to exogenous NE.[11]

An improved prognosis for hypertensive patients may depend on the type of drug used for treatment in addition to good blood pressure control. Thus, the incidence of fatal and non-fatal coronary heart disease is reported to be significantly lower in patients treated with a β-blocker than in a control group.[12] However, use of propranolol, in combination with a thiazide, reduces serum high density lipoprotein cholesterol and increases serum triglycerides and urate more than methyldopa and thiazide, or thiazide alone; these biochemical changes are considered to be risk factors for coronary heart disease.[13] In rats, vasodilators such as minoxidil cause cardiac hypertrophy which may be prevented or reversed by methyldopa but not by propranolol.[14] Increased collagen biosynthesis, due to high blood pressure, causes structural changes in blood vessel walls leading to an increased wall/lumen ratio and alteration of vascular elasticity which is difficult to reverse;[15] chlorothiazide and reserpine prevent or reduce the collagen formation.[16]

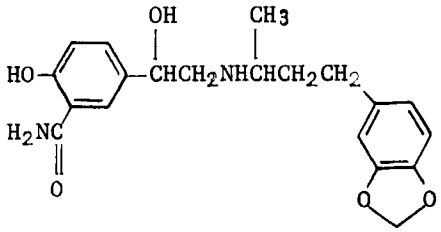
Adrenergic Blocking Agents - An excellent review of the use of β-blocking agents in hypertension has appeared.[17] Propranolol, the sole β-blocker available in the U.S. since 1967, has now been joined by the cardioselective agent metoprolol (Lopressor®) which was approved for use in hypertension in August, 1978.[18] A second β-blocker, timolol (Timoptic®), also received FDA approval but only for the treatment of glaucoma.[19] Animal

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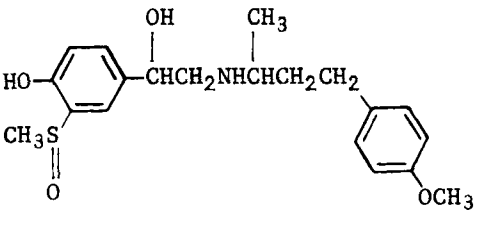
studies on nadolol show no evidence of toxicity or carcinogenicity[20] and this agent, as well as pindolol, is expected to receive marketing approval soon.[21] The use of β-blockers in hypertension and other disease states is discussed in Chapter 9.

All of the β-blockers now in common clinical use worldwide seem to be effective in lowering blood pressure but the mechanism of their antihypertensive action is still uncertain.[17] Although β-blockers inhibit renin release from the kidney, probably by an action on β_1-receptors[22,23] there is generally only a poor correlation between decrease in plasma renin activity (PRA) and blood pressure decrease during therapy. Recent studies show a much better correlation with sympathetic responsiveness[24] or with decrease in sympathetic nerve activity (SNA).[25-28] This decrease in SNA may be an indirect reflex effect due to dampening sensory input to the CNS from the heart or to a direct central action. There is now good evidence for the existence of central β-receptors,[28,29] but only indirect evidence for their involvement in the antihypertensive action of β-blockers. Direct injection of β-blockers into the brain results in blood pressure lowering but this is not necessarily due to blockade of β-receptors.[30,31] The antihypertensive potency of six β-antagonists in man correlates significantly with their ability to penetrate into rat brains, but this is not conclusive evidence for a central mechanism of action.[32]

A vasodilating action of the β-blockers alprenolol,[33] propranolol[34] and oxprenolol[35] has been demonstrated but is probably not significant at therapeutically useful doses. In contrast, the hypotensive action of labetolol is primarily due to a reduction in peripheral vascular resistance due to its substantial α-blocking component in addition to a β-blocking action.[36] The actions of labetolol at the membrane level have been demonstrated. This drug inhibits the binding of the specific ligands (-)-[^{3}H]-dihydroalprenolol and [^{3}H]-dihydroergocryptamine to β- and α-adrenoceptors of rat heart and liver preparations and has a 10-fold higher affinity for the β- than for the α-receptors.[37] The pharmacology and therapeutic use of labetolol has been reviewed.[38] A structural analogue of labetolol, RMI-81968 (1), is reported to have a direct vasodilating action in addition to α- and β-blocking actions.[39] A similar structure, WIN-40,808-7 (2), exhibits both β-antagonist and direct vasodilating actions but no α-blockade. It lowers blood pressure in renal hypertensive dogs at 25 mg/kg/day with little change in heart rate.[40]



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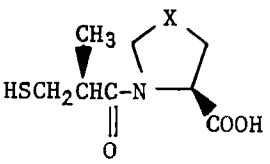


2

The vasodilating and β-blocking action of 4-(2-imidazolyl)phenoxy propranolamines was reported last year. Additional studies show that substitution *ortho* to the imidazoline group reduces both the direct vasodilator and intrinsic sympathomimetic potency.[41]

The use of α-blockers in the treatment of hypertension has long been in disrepute However, the discovery of the unique α-blocking actions of prazosin has generated renewed interest in this form of treatment. The pharmacological and clinical effects of this drug have been reviewed.[42] In comparison to phentolamine in animals, prazosin is a more potent hypotensive agent, it has less potential for orthostatic hypotension and is more selective for post-synaptic α-receptors.[43] The lack of a direct vasodilator action for prazosin has been confirmed in rats, rabbits, and dogs.[44,45] Considerable efforts continue to try to explain the absence of reflex cardiac and renin effects after this drug. These effects are generally attributed to prazosin's selective blockade of post-synaptic α-receptors as has been shown in rabbit pulmonary artery and in rats.[46,48] However, in dogs and cats, this selectivity for post- over pre-synaptic receptors is much less and may not explain the lack of cardiostimulation in these species and in man.[49,50] Alternatively, the lack of tachycardia may be due to inhibition of the enzyme dopamine β-hydroxylase.[51] Clinical studies with the prazosin analogue, trimazosin, and the α-blocker, indoramine, have demonstrated antihypertensive actions in patients, without tachycardia.[52,53] The latter drug shows selectivity similar to prazosin for post-synaptic α-receptors.[54,55]

Inhibitors of the Renin-Angiotensin System - This area was reviewed in detail in the previous Annual Reports. Other useful reviews on the renin-angiotensin system (RAS),[56,57] factors influencing renin release,[58] the rationale and SARs for angiotensin converting enzyme (CE) inhibitors,[59] and animal pharmacology[60] of captopril have appeared. Of major interest during the past year has been the first reports of chronic treatment of hypertensive patients with the orally active CE inhibitor, captopril (3).[61-64] As expected, this drug is very effective in renovascular, malignant, and essential hypertensives characterized by high renin levels. Surprisingly, however, it is also effective in patients with normal renin levels and even in some low renin patients. The doses of captopril used in these studies were 50-100 times those previously shown to be required to block the conversion of angiotensin I to angiotensin II, so inhibition of the CE may not completely account for its antihypertensive action.[65]



3 X=CH_2

4 X=S

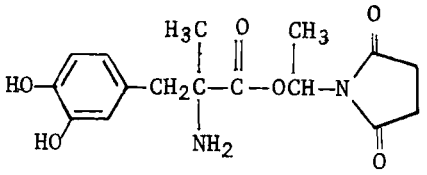
Captopril was well tolerated by most patients but rash and fever occurred in some patients. Patients with very high initial PRA may be extremely sensitive to blockade of the RAS and at least one episode of symptomatic hypotension has occurred after captopril in a volume-depleted patient.[61] These clinical studies, as well as animal studies,[60] generally support the view that the major mechanism of the antihypertensive action of captopril is by suppression of the RAS with consequent decrease in angiotensin II. However, potentiation of the physiological vasodilator bradykinin, from renal or extrarenal sources, may also contribute to its action.[66,67] In fact, the hemodynamic effects of captopril are similar to those produced by vasodilators that act on both veins and arteries.[68]

First reports on the pharmacokinetics of captopril in man indicate that it is well absorbed orally and is excreted largely unchanged with a half-life of 0.9 hr.[69] The disulfide is the only metabolite identified so far in man and in mice.[69,70]

A new orally active CE inhibitor, SA-291 (4), is the most potent of a series of thiazolidine analogues of captopril.[71] The SAR appears similar to that found for captopril and SA-291 shows the same spectrum of activity as captopril in animals.[72]

The nonapeptide teprotide (SQ 20,881) also inhibits CE but it has limited clinical potential since it is not orally active. However, this drug is very useful for diagnostic purposes in renin profiling and for emergency treatment of patients with malignant hypertension.[56] It has been used successfully, by *i.v.*, *i.m.*, and *s.c.* administration for 4 months to treat high renin hypertension resistant to other drugs.[73] The angiotensin II antagonist saralasin ('Sar-^{8}Ala-ang II) is also useful for renin profiling but is less predictable than teprotide because of its partial agonist action.[56] Saralasin is only effective in lowering blood pressure directly dependent on pretreatment PRA. This drug may increase pressure in some patients or cause severe hypotension in patients strongly dependent on angiotensin II for blood pressure maintenance.[74,75] Comparison of teprotide and saralasin in salt-depleted rats failed to show any difference in their blood pressure lowering effects and no additional lowering occurred when they were administered together, suggesting that both function specifically to directly, or indirectly, prevent vasoconstriction due to angiotensin II.[76] An interesting hypothesis proposes that the blood pressure decrease, following CE inhibitors or angiotensin II antagonists, is caused by a vasodilator action of renin itself on vascular walls. This action is only unmasked when opposing vasoconstrictor effects of angiotensin II are blocked.[77]

Centrally Acting Drugs - Evidence, supporting the view that drugs such as clonidine and α-methyldopa regulate blood pressure by an action on central α-receptors, has been reviewed.[78] A survey of clinical studies on α-methyldopa show that it causes very favorable hemodynamic effects compared to β-blockers; it lowers blood pressure mainly by decreasing total peripheral resistance with little effect on heart rate or cardiac output and usually a decrease in PRA.[79,80] In a large group of patients treated with methyldopa, the major side effects were excessive hypotension and drowsiness.[81] A prodrug 5 was 3.2 times as potent as methyldopa in SH rats, probably due to better oral absorption, but this increased potency did not carry over to man.[82]



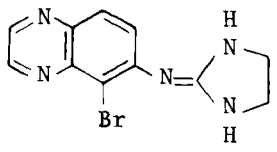
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The clinical dose of clonidine is about 0.3-1.5 mg/day and patients may be resistant to its actions at higher doses, probably due to its α-stimulant, vasoconstrictor action opposing the central depressor effects.[83] After *i.v.* injection of this drug in patients to give plasma levels of 0.1-2.2 ng/ml, clonidine caused dose-related decreases in blood pressure; however, at plasma levels greater than 10 ng/ml no hypotensive action was observed.[84] Similarly, in rats, clonidine caused dose-related decreases in blood pressure at 1-10 μg/kg *i.v.*, but no additional effect was found at higher doses.[85] Plasma levels of clonidine after single or multiple doses also correlated well with sedation and dry mouth caused by the drug.[86] Metabolites probably play little part in clonidine's effects since it is excreted mainly unchanged in man; however, in rats, and

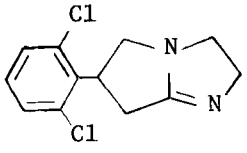
especially in dogs, it is extensively metabolized by hydroxylation in the aromatic and imidazoline rings.[87]

Rebound hypertension occurs in a small percentage of patients after sudden cessation of clonidine therapy.[88] Recent studies in man[89,90] and rats[91] support the view that this effect is caused by release of norepinephrine from sympathetic nerves,[90,91] probably as a result of removing the dampening influence of clonidine on central noradrenergic neurones. A similar central noradrenergic excitation may be involved in the opiate withdrawal syndrome which is prevented by low doses of clonidine, possibly by replacing opiate-mediated inhibition of central noradrenergic neurones with α_2-adrenergic inhibition in areas such as the locus coeruleus.[92]

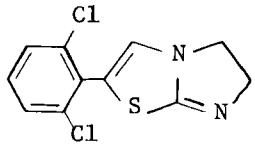
Most studies support the view that clonidine and similar drugs exert their antihypertensive action by stimulating central medullary α-adrenoceptors. Clonidine appears to act directly on the α-receptors since its action in rabbits is not affected by depleting central noradrenergic neurons with 6-hydroxydopamine.[93] These receptors are probably of the presynaptic or α_2-type and clonidine's actions are blocked by the selective α_2-blocker mianserine.[94] Clonidine's sedative action also appears to be mediated by central α_2-like receptors.[95,96] Several alternative mechanisms have been proposed to explain clonidine's actions. It may lower blood pressure and suppress sympathetic activity by stimulating inhibitory serotonin receptors on sympathetic preganglionic neurones at the spinal level of sympathetic integration.[97] Central histamine H_2-receptors may be implicated in clonidine's antihypertensive action since it stimulates H_2-receptors in guinea pig atria[98,99] and injection of the antagonists cimetidine or metiamide *i.c.v.* blocked clonidine's hypotensive and bradycardic actions in cats.[100] However, the action of metiamide may be due to an α_2-blocking action.[101]



6



7



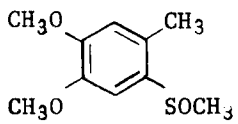
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The close clonidine analogue, tolonidine, was introduced to the French market during the past year. It is similar to clonidine but no rebound hypertension has been reported.[102] Clinical studies on guanabenz,[103,104] lofexidine,[105] guanfacine,[106-109] and UK-14,304 (6),[110] show hypotensive activity and side effects similar to clonidine. The hemodynamic effects of guanfacine in rats are very similar to clonidine but it decreases heart rate and PRA more and causes greater rebound hypertension and tachycardia at equal hypotensive doses.[111] Contrary to earlier expectations based on animal studies, this drug does cause sedation and dry mouth as well as rebound hypertension after sudden termination of therapy.[107,108] A new clonidine-like drug, 7, ICI-101,187, is reported to be as potent as clonidine in lowering blood pressure in animals but is only one-tenth as sedating.[112] Structurally this drug is quite similar to 8 (Sandoz 44-549), which is an even more potent hypotensive agent than clonidine in rats.[113]

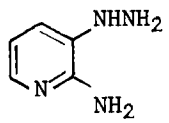
Like clonidine, the interesting drug R-28935 lowers blood pressure by centrally mediated depression of sympathetic nerve function.[114] In contrast, however, it is suggested that the action of this drug is due to inhibition of central neurotransmission by blockade of postsynaptic α-receptors.[115] The actions of the drug urapidil are complex but it appears to both stimulate presynaptic and block postsnaptic α-receptors.[116]

Antihypertensive Vasodilators - A common characteristic of hypertensive patients, regardless of the etiology of their disease, is abnormally high peripheral vascular resistance. Drugs that act directly on peripheral vasculature to decrease resistance are, therefore, logical agents for the treatment of hypertension. Unfortunately, sympathetic reflex actions leading to cardiac stimulation, hyperreninemia and fluid retention limit the hypotensive action of vasodilators and they are used mainly in combination with β-blockers and diuretics for the treatment of more severe hypertension.[117-120] A vasodilator that may not cause undesirable reflex cardiac effects is the calcium antagonist verapamil. This drug lowered blood pressure without tachycardia in one study[121] but was ineffective and poorly tolerated in another.[122] A useful classification of hypotensive vasodilators, which explains their different hemodynamic profiles, has been described. These agents cause arteriolar dilatation but differ in their venodilating and sympathetic stimulant actions.[123] Of six vasodilators studied in hand veins and forearm arteries of volunteers, only hydralazine was a selective arterial dilator.[124]

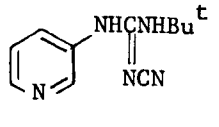
Various prostaglandins (PGs), such as PGE_2, and the recently discovered prostacyclin (PGI_2), are potent, short-acting, renal and systemic vasodilators that may have a physiological role in the control of blood pressure.[125-127] Increasing the intake of linoleic acid, the dietary precursor fatty acid of endogenous PG biosynthesis, lowered blood pressure of mild hypertensives.[128] The hypotensive action of the PG precursor arachidonic acid in rats depends on a metabolite since its effect is blocked by endoperoxide cylooxygenase enzyme inhibitors.[129] The PG system may also be involved in the antihypertensive action of hydralazine and diazoxide. These drugs are reported to selectively inhibit the formation of the potent, vasoconstrictor, metabolite of arachidonic acid, thromboxane A_2 (TxA_2), from the endoperoxide PGH_2, and may stimulate PGI_2 production.[129,130]



9



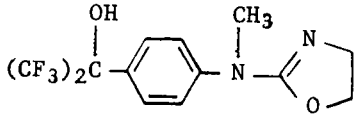
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11

A number of interesting new antihypertensive vasodilators have been reported during the past year. Tolmesoxide (9) is similar in potency to diazoxide in DOCA hypertensive rats. It reduced blood pressure by 25-45% at doses of 20-100 mg/kg *p.o.*[131] and was equally active as a vasodilator in veins and arteries of volunteers.[124] The hydrazinopyridine EU 2540 (10) is a potent hypotensive agent in SH rats but causes intense tachycardia.[132] The cyanoguanidine 11 is 150 times as potent in SH rats as the clinically used agent guancydine; the rationale for the synthesis of this

compound was based on the previously observed hypotensive activity of the bioisosteric pyridylthiourea.[133] Compound 12 (Sch 21700) is the most potent of a new structural class of vasodilators. It is 11 times as potent as hydralazine in decreasing the blood pressure of SH rats.[134]



12

The mechanism by which vasodilators cause vascular relaxation is still poorly understood. Hydralazine inhibits contractions of isolated rabbit aortic strips and may act by restricting calcium entry into K^+ depolarized cells. *In vitro*, as well as *in vivo*, the acetone hydrazone metabolite and the butanone hydrazone are more potent vasodilators than the parent compound.[135] Another major hydralazine metabolite, the pyruvic acid hydrazone, was not hypotensive in rabbits.[136] The action of hydralazine on rat arteries is blocked by adenosine triphosphate (ATP) and it is suggested that this drug interacts with specific receptors physiologically activated by ATP or a related substance released from sympathetic nerve terminals.[137] Studies of three vasodilators in rat vas deferens failed to confirm the hypothesis that cyclic GMP is a mediator of vascular smooth muscle relaxation.[138] The vasodilating action of dihydropyridines, such as nifedipine, is generally attributed to their calcium antagonist action. However, the structurally similar drug YC-93 and various analogues are extremely potent inhibitors of cyclic AMP phosphodiesterase, leading to substantial increases in intracellular cyclic AMP which may be at least partly responsible for their vasodilating properties.[139]

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Chapter 8. Antithrombotic Agents

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Introduction - Though the death rate due to vascular diseases has peaked and slightly decreased in the past 2-3 years, they are still the number one cause of death in the United States and in the total western civilization. It has been suggested that the death rate has decreased due to better early care after a heart attack or stroke and/or because people are taking care of themselves due to their awareness of the risk factors that relate to the development of vascular disease. Though no statistics are available, the number of people who have vascular disease has probably not decreased; only the frequency of acute attack and death rate has been affected.

Thrombosis or blood coagulation components play a role in the development of vascular disease and in most cases, thrombosis is a part of the death-related event. Several good reviews, have been published in which interrelationships of atherosclerosis and thrombosis and other vascular interplays are discussed.[1-4] Murano has reviewed the relationship of activation of coagulation and Hageman Factor with lysis and kinin activity,[5] and Berghaus has reviewed the relationship of complement to blood coagulation.[6]

Therapy for the control or prevention of the acute events in death due to vascular disease can be approached in several ways. One general approach would be to control the interaction of blood components with the diseased blood vessel or with each other. In both these cases antithrombotic agents of diverse mechanism should be helpful. Whether control of fibrin formation, fibrinolysis or platelet function is desirable, each patient's problem would dictate the therapy best suited. Several reviews are available in which the different blood coagulation mechanisms that may be helpful in such therapy are discussed.[1,7-9] Reviews have also been published in which methodology is discussed for the testing of compounds[10] and for the determination of abnormalities in platelet function.[11]

This review will report on the progress made in understanding the biochemistry and physiology of blood coagulation and thrombosis and the development of active antithrombotic agents in the last 2 years. It is suggested that the reader refers to the most recent review of this series.[12]

Platelet Aggregation Inhibitors

Since anticoagulants such as heparin and vitamin k antagonists have not been found to effectively control arterial thrombosis, the main attention for the past 15 years has been to study platelet function and develop compounds to control the role of the platelet in arterial thrombosis, mainly through the control of platelet aggregation or white thrombus formation. The greatest advance in the last several years has been the discovery, structure determination and biological character-

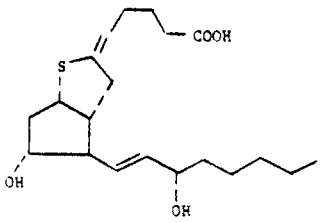
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ization of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2). These two compounds along with PGE_2 and $PGF_{2\alpha}$ have common precursors, the endoperoxides PGG_2 and PGH_2. TXA_2 (synthesized in the platelet) induces platelet aggregation, while PGI_2 (synthesized in the normal blood vessel) inhibits platelet aggregation. Several excellent reviews on this subject,[13-15] as well as general reviews and mechanistic articles on the role of the platelet in thrombosis have been published.[16-22] (cf. also chapter 17 in this volume). The relationship of diabetes mellitus to abnormal platelet function due to an increased sensitivity in the second phase of platelet aggregation resulting from TXA_2 synthesis, has been reviewed by Colwell and associates.[23,24]

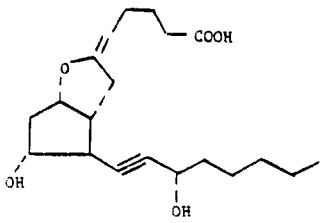
There are at least 3 different mechanisms by which platelets will aggregate; 1) by primary aggregation induced by ADP, 2) one of the aggregation pathways induced by thrombin and 3) one induced by the production of TXA_2. Inhibitors of platelet aggregation may inhibit the inducers directly, inhibit their production or interfere with the biochemical process involved in platelet aggregation. The inhibitor may be a specific (direct) one or one that affects a modifier of one of the biochemical steps involved in aggregation (indirect). The final effect may be on the flux of calcium in or out of the cell or its membranes. Thromboxane A_2 has been shown to be a calcium ionophore.[25-27] Calcium ionophores such as A23187 and X537A cause platelet aggregation.[28-30] Calcium fluxes and distribution are affected whether the stimulus for aggregation is collagen or epinephrine, thrombin, serotonin, or ADP, that either produces TXA_2, lowers cyclic AMP levels, or "stabilizes membranes."

The compounds that have been reported to influence platelet aggregation recently will be discussed under their respective classification as follows: 1) prostaglandins and synthetic analogs, 2) non-steriodal antiinflammatories (PG synthesis inhibitors) and 3) miscellaneous.

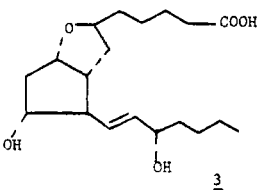
Prostaglandins and Synthetic Analogs (Also see Chapter 17 of this volume.) Before the discovery of PGI_2, the prostaglandins PGE_1 and PGD_2 were considered to be the PG's of choice for inhibition of platelet aggregation. Since they were biologically unstable, synthetic derivatives that would be more stable were considered. Now that PGI_2 has been shown to be the most potent inhibitor[31-33] and is active when given intravenously[34-36], more stable derivatives of PGI_2 are being synthesized. Such more stable derivatives as 6, 9-thiaprostacyclin (1),[37] 13, 14-dehydro PGI_2 (2)[38] and 5, 6 α dihydro PGI_2 (3)[39] have been synthesized and tested.



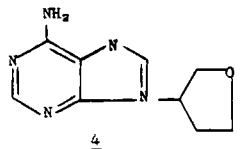
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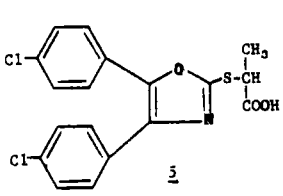
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It has been suggested that PGI_2 inhibits platelet aggregation by the stimulation of adenyl cyclase and the increase of cyclic AMP levels.[40,41] This is the opposite effect of most aggregating agents which usually decrease cyclic AMP levels. A compound SQ 22536 (4),[42] has been reported to inhibit adenyl cyclase and lower cyclic AMP levels. This compound does not aggregate platelets, but does increase platelet aggregation when challenged with an aggregating agent.[42] This supports the theory that cyclic AMP regulates platelet aggregation.

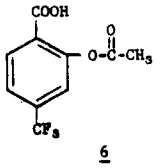
Aspirin and other Non-steroidal Anti-inflammatory Compounds - Most non-steroidal anti-inflammatory (NSAI) compounds including aspirin inhibit the cyclooxygenase that catalyzes the formation of the endoperoxides PGG_2 and PGH_2 from arachidonic acid. Normally this would inhibit both the synthesis of TXA_2 and the second phase of platelet aggretation, but it would also inhibit PGI_2 formation as it is also derived from PGG_2 and PGH_2. The inhibition of PGI_2 formation is considered to be undesirable because it is a potent inhibitor of platelet aggregation. TXA_2 is synthesized in the platelet, while PGI_2 is formed in the blood vessel wall. Aspirin usage has been questioned for this reason. Aspirin affects the platelet at lower doses and for a longer time than it affects other tissues. Therefore low dose aspirin is useful.[43] However, experiments have shown that low dose aspirin (< 15 mg/kg) increases bleeding time while higher doses (> 50 mg/kg) do not affect the bleeding time.[44,45] Several clinical studies have been conducted that support the conclusion that aspirin is helpful in reducing the frequency of transient ischemia attacks (TIA) in men.[46,47] Women were not protected by aspirin treatment.[48] The explanation for this sex difference is undetermined.

Additional data were reported on the following previously mentioned NSAI compounds: sulfinpyrazone,[49,50] naproxen,[51] ibuprofen,[51] indomethacin,[52] halofenate,[53] and flurbiprofen.[54] Sulfinpyrazone was not shown to have a beneficial effect on patients in the Canadian aspirin-sulfinpyrazone stroke study.[47] However, in a progress report of the multi-center myocardial infarction (MI) study with sulfinpyrazone, it was reported to greatly decrease mortality of those patients that had had an MI.[55,56] Halofenate was reported not to act at the cyclooxygenase step since it does not inhibit arachidonic acid-induced platelet aggregation.[57] However, it does inhibit epinephrine and collagen-induced aggregation, leading to the suggestion that it inhibits the earlier step in which phospholipase A_2 hydrolyses arachidonic acid from phospholipid.[57]

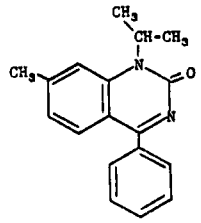
Compounds that have recently been reported to inhibit the cyclooxygenase are: tioxaprofen (EMD 26644) (5),[58,59] triflusal (6),[60] proquazone (7),[61] diflunisal (8),[62] sulindac (9) (the sulfide metabolite of sulindac which is the active species of sulindac in man, is 8 times more potent than aspirin),[63] diflumidone (10),[64] pranoprofen (11),[65] and alclofenac (12).[66]



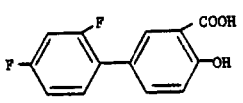
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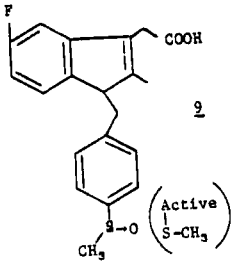
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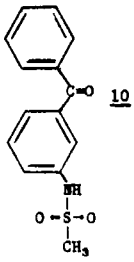
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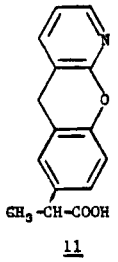
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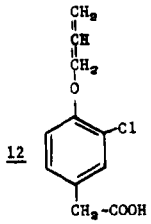
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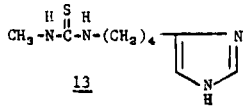


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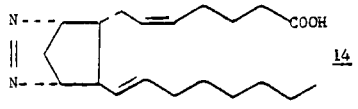


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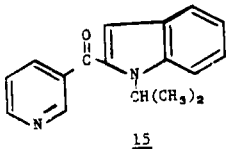
Since NSAI compounds inhibit the formation of PGG_2 and PGH_2, their effectiveness is due to the decrease in TXA_2 formation. It may be more desirable to specifically inhibit TXA_2 synthesis or activity, so that PGI_2, PGE_2 and $PGF_{2\alpha}$ can still be synthesized unimpeded. TXA_2 has caused MI when injected into rabbits.[67] It also has been reported that TXA_2 may react with the same receptors in the brain, as those to which benzodiazopines react.[68] Compounds that were reported to specifically block TXA_2 synthetase are: burimamide (13),[69] 9,11-azo prosta-5,13-dienoic acid (14),[70] L8027 (nictindole) (15),[71] 15-deoxy-9,11-(epoxyimino) prostaglandins (16),[72] imidazole[73,74] and 1-methyl imidazole[74] (17, 18). In a series of 1-alkyl imidazole derivatives, 1-nonyl (19) was the most potent inhibitor and an SAR was evident.[75] Nicotinic acid has also been reported to inhibit TXA_2 synthesis.[76] More work has also been done on N-0164.[77] Phthalazine (EG626) (20) has been reported to inhibit the activity of TXA_2, but not its synthesis.[78-80] Some recent evidence suggests that dipyridamole, previously reported to inhibit phosphodiesterase, inhibits TXA_2 synthesis.[82,83]



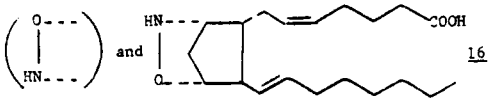
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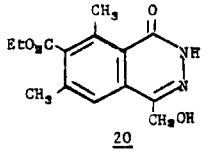
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R = H (17)
R = CH_3 (18)
R = $(CH_2)_8$-CH_3 (19)



20

Miscellaneous Compounds - Several compounds previously reported were further investigated as inhibitors of platelet aggregation and new data reported. These were carbenicillin and ticarcillin,[84] BL3459,[85] ticlopidine,[86] suloctidil,[87-89] and vinca alkaloids.[90-91]

A metabolically more stable derivative of BL3459, BL4162 (21), was found to inhibit collagen-induced platelet aggregation similar to that of BL3459.[92,93] It is now undergoing clinical evaluation.

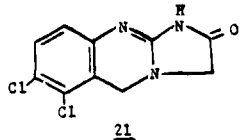
Methaqualone (22), a sedative-hypnotic, was reported to inhibit both ADP and collagen-induced platelet aggregation *in vitro* in human platelet-rich plasma and *in vivo* in rabbits.[94]

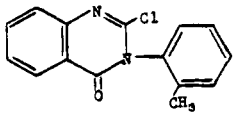
Pentoxifylline (23), a methyl xanthine derivative, was reported to inhibit both ADP and serotonin-induced platelet aggregation in the stump-tail monkey.[95]

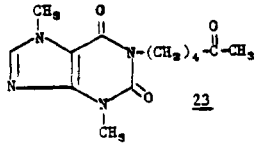
Mianserin (ORG GC94) (24), a clinically effective antidepressant suggested as therapy for migraine, has been found to inhibit platelet aggregation, especially when induced by serotonin.[96]

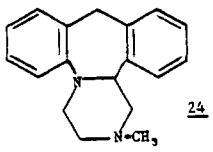
Several flavanoids were reported to affect platelet adhesiveness in repeatedly bred rats.[97] The order of potency for 6 different flavanoids were determined. Venoruton P_4 (25) was the most potent, while rutin (26) was much less potent.

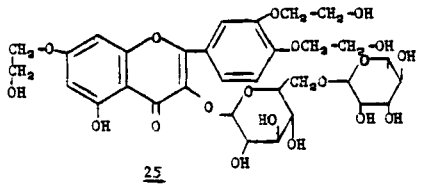
Since the original classic work of Born in 1963 on adenosine and 2-chloroadenosine, many adenosine derivatives have been reported to inhibit platelet aggregation, especially induced by ADP. In 1977 work was reported on some new derivatives of S-substituted 2-thioadenosines (27) and N-substituted 2-aminoadenosines (28).[98]

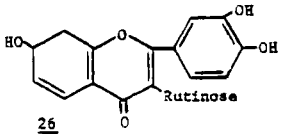

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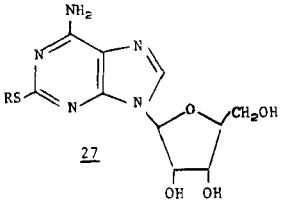

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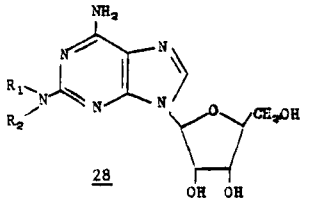

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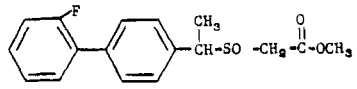

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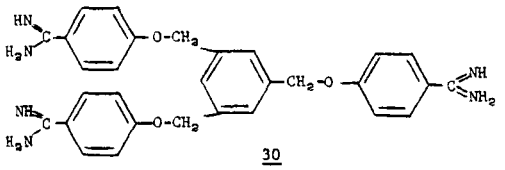

28

A new compound called SH 1117 (29) has been reported to inhibit platelet aggregation and be synergistic with aspirin similar to dipyridamole but without its cardiovascular effects.[99]

Many papers have been published reporting the serine protease inhibitory activity of amidine or diguanide derivatives (see chapter 21 of this volume). A series of aromatic amidine compounds was reported to inhibit platelet aggregation induced by ADP, collagen and thrombin. The most active compound was (30).[100]



29



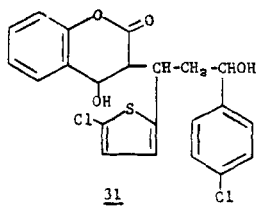
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Anticoagulants

During blood coagulation either an intrinsic (all blood) system or an extrinsic (tissue juice-lipoprotein) system is activated. In either case the pathways meet at the activation of Factor X, forming a proteolytic enzyme Factor X_a. This enzyme in the presence of cofactors (calcium ion, phospholipid and Factor V) will form thrombin from prothrombin. Heparin is a cofactor for a protein called antithrombin III which circulates in the plasma and is an inhibitor of both Factor X_a and thrombin. Antithrombin III neutralizes these 2 enzymes by molecular combination; heparin increases the rate of this neutralization. In the past 2 years work has continued on the mechanism of blood coagulation.[101-103] More data have also been reported on the effectiveness of mini-dose heparin.[12,104,105]

Due to the lowered level of antithrombin III found in some families[106], or during treatment with heparin, it has been suggested that the intravenous administration of purified anti-thrombin III would be beneficial.[107]

Since heparin is a hetereogeneous mixture that varies markedly depending on the source, further investigations on fractions and relative activities are in progress.[108,109]



31

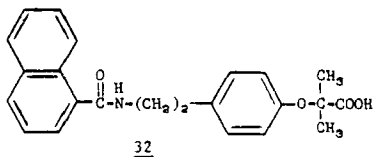
A semi-synthetic heparinoid, A73025, which is a glycosminoglycan polysulfate of bovine origin with an average molecular weight about 1/2 that of heparin has a potency of about 90 μ/mg based on mucosal heparin. It produces good antithrombin III activation, but has little effect on overall clotting. It is suggested to have good potential as a heparin substitute.[110]

The action and properties of tioclomarol(31), an anti-vitamin k -coumarin type compound, has been reviewed.[111]

Fibrinolytic Agents

Fibrinolysis has recently been reviewed.[1,12,112] In order for fibrinolysis to occur, the proenzyme plasminogen must be activated to plasmin by an activator. Several activators can be formed; one from Hageman factor components after its activation, or by a tissue activator released from normal blood vessels. Urokinase is an activator considered to be limited in its function to the kidney. Streptokinase, a single-chain protein from β-hemolytic streptococci, is an activator that has been studied for many years and has found clinical use especially in Europe. Its main weakness is decreased potency due to antibody formation. The use of urokinase, which is not antigenic, has been limited due to supply problems and cost of manufacture from urine. However, the problem may have been solved due to a new source from kidney tissue culture (Abbokinase®).[12]

Little new work has been done in this area. The isolation and testing of a low molecular weight species (L-UK) and a high molecular weight species (H-UK) of urokinase has been reported.[113] The H-UK had the greater activity. Further work has been reported on urokinase and its successful use.[114]



32

The fibrinolytic activity of the onion and garlic constituent cycloalliin has been confirmed.[115] Aspirin has been reported to have fibrinolytic activity, independent of its platelet effects.[116] A heparin-urea complex was reported to enhance fibrinolysis,[117] while each component separately had no effect.

Bezafibrate (32) was reported to shorten euglobulin lysis time in human patients.[118]

Comment - Many different clinical trials are in progress on a variety of antithrombotic agents (antiplatelet, heparin, and thrombolytic agents).[119] In the past 2 years progress has been rapid. Aspirin has proven to be effective in reducing the frequency of TIA. Sulfinpyrazone may reduce the death rate after MI. An inhibitor of platelet aggregation (BL 4162) developed specifically for this activity, and not already under evaluation or marketed for another indication, is being tested clinically. The future is indeed bright that some of these compounds will be useful in the control of thrombosis, which will stimulate further development of more potent and safer drugs and tools to enable us to understand the mechanisms involved in thrombosis and vascular disease.

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Chapter 9. β-Adrenergic Receptor Blockers as Therapeutic Agents

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Introduction - Ahlquist's concept which classified adrenergic receptors[1] into two distinct groups provided the basis for the emergence of a class of drugs which has made substantial impact on the understanding and the treatment of a wide order of disease states. During the early era of β-blockers, their initial proposed therapeutic use was in the treatment of angina pectoris. It was thought that the β-receptor mediated increases in heart rate and myocardial contractility obtained with catecholamine release were detrimental in patients suffering from ischemic heart disease because these actions increased oxygen demand in the presence of a limited oxygen supply. The first β-blocker to be effective in the treatment of angina pectoris was pronethalol.[2,3] Shortly after the introduction and subsequent withdrawal of pronethalol from clinical trials because of carcinogenicity,[4] propranolol was developed.[5] Propranolol was extensively evaluated and was shown to be beneficial in the treatment of angina pectoris.[6] This drug has since become one of the most widely investigated and widely used β-blocking agents.

At the same time β-blockers were being considered as anti-anginal agents, other therapeutic uses in cardiovascular diseases were also being considered. In animal studies, observations were made that pronethalol induced bradycardia and thereby suggested the use of β-blockers in the treatment of arrhythmias.[2] Dichloroisoproterenol and pronethalol were demonstrated to possess antiarrhythmic activity against experimentally-induced cardiac arrhythmias;[7,8] subsequently, pronethalol was reported to possess antiarrhythmic efficacy in humans.[9] Similar activity was observed with propranolol.[10,11] β-Blockers were also considered for use in treatment of myocardial infarction. The first clinical trial of propranolol in myocardial infarction demonstrated that β-blockers reduced mortality in this patient population.[12] The observation that propranolol lowered blood pressure in patients who were being treated for angina[13] and the significant antihypertensive activity obtained in clinical trials with propranolol[14,15] led to the use of β-blockers as primary drugs in the treatment of hypertension. Numerous β-blockers have been developed and additional therapeutic indications have been suggested for them since the initial development of pronethalol and propranolol as clinically useful agents in treatment of various cardiovascular disorders. The purpose of this report is to present an update on the status of this class of agents.

A list of β-blockers indicating their structure and developmental status and the absence or presence of β-receptor selectivity, partial agonist activity and non-specific cardiac depressant property, is presented in Table I for reference. In addition to propranolol, two new β-blockers have now been approved for use in man in the U.S. Metoprolol has been approved for use in hypertension,[16] timolol has been approved for use in glaucoma.[17] In other parts of the world, there are at least 17 β-blockers available for use.[18]

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Pharmacologic Properties - By definition, all β-blockers block β-adrenergic receptors. It is this action which is responsible for their efficacy in various clinical conditions.[19] Some β-blockers have selectivity in blocking sub-types of β-receptors. Cardioselectivity (β_1-receptor function is inhibited to a greater degree than β_2-receptor mediated responses) appears theoretically to be of clinical significance. In addition to claimed benefit in asthmatic patients,[20,21] cardioselective β-blockers are proposed to offer an advantage over non-selective ones in patients with peripheral circulatory insufficiency.[22,23] Theoretically, tissue blood flow would be less impaired with these agents because of reduced effects on vascular β_2-receptors. β_1-Selectivity may also beneficially influence the effects that β-blockers have in diabetic patients. The prolongation of the insulin-induced hypoglycemia which is observed with β-blockers is reported to be less with cardioselective β-blockers. In addition, the hypertensive response which occurs secondarily to insulin-induced hypoglycemia in the presence of β-blockade has been reported to be less severe with cardioselective than with non-selective β-blockers.[24] The actual significance of receptor differentiation in terms of common clinical practice may be questionable, however, because such selectivity occurs with low doses and it is lost with higher doses which are commonly used in patients.[25,26] Furthermore, the theoretical advantage of selective β-blockers relies on the assumption that there is an absolute organ separation of β_1 and β_2 receptors.[27] Such an assumption is tempered by evidence which suggests that a given organ possesses both β_1 and β_2 receptors, the relative proportion varying from organ to organ.[28,29]

A different type of selectivity than that afforded with agents blocking preferentially β_1 as compared to β_2 receptors has been suggested for mepindolol. This agent has been claimed to block chronotropic β_1 receptors to a greater extent than inotropic β_1 receptors.[30,31] The presumed advantage of this type of agent is that it would be accompanied by a lower risk of cardiac decompensation than that associated with other β-blockers. Again, such selectivity may be lost with commonly used clinical doses.

Controversy still exists with reference to the non-specific cardiodepressant action of β-blockers associated with their membrane stabilizing properties. This action is directly related to dosage levels[19] and it may be relevant at high doses encountered in clinical practice. Furthermore, pathological as well as other factors can alter the pharmacokinetics of these agents and influence clinical response.[25,32-34] Membrane stabilizing activity of β-blockers appears to be directly correlated with their lipid solubility.[25] Moreover, absorption, distribution and biotransformation correlate with lipid solubility.[18,35] β-Blocking agents which are more lipid soluble cross the blood brain barrier more readily and therefore cause such side effects as drowsiness, depression, hallucinations and vivid dreams.[25] The lipophilic β-blockers also have the capacity to cross the placenta and can cause perinatal complications.[36,37]

TABLE I. β-Receptor Blocking Agents

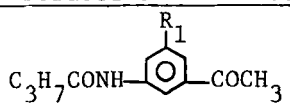
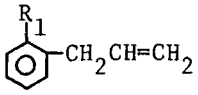
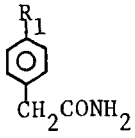
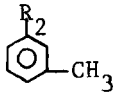
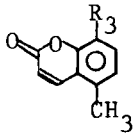
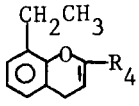
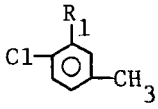
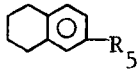
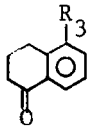
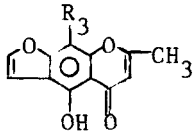
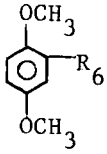
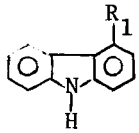
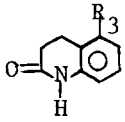
Generic Name (Trade)	Code	Structure	Status[a]	Cs[b]	PA[c]	MSA[d]	Ref[e]
Acebutolol (Sectral)	M&B 17803A		M	+	-	+	38
Alprenolol (Aptin)	H 56/28		M	-	+	+	39
Atenolol (Tenormin)	ICI 66082		M	+	-	-	40
Bevantolol (Bevantol)	CI 775		C	+	-	+	41
Bucumolol	CS 359		C	-	-	+	42
Bufuralol	Ro 34787		C	-	+	+	43
Bupranolol (Betadrenol)	K1 255		M				44
Butidrine (Recetan)	CO 405		M	-		+	45
Bunitrolol[f] (Stresson)	Ko 1366		M				46
Bunolol	W 6412A		C	-	-	+	47
Butocrolol	P 18		E		-	+	48
Butoxamine[f]	BW 64-9		E	-	-		49
Carazolol	BM 51052		C		-		50
Carteolol	OPC 1085		E		+		51

Table I (cont.)

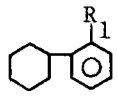
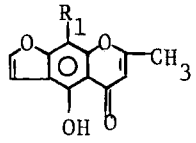
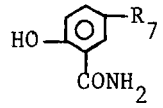
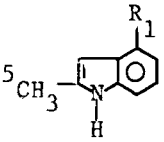
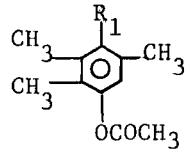
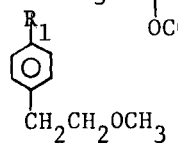
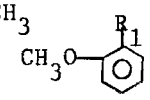
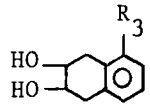
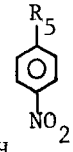
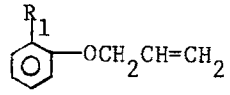
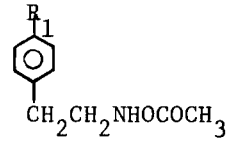
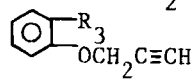
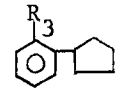
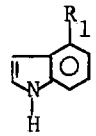
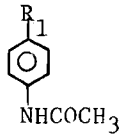
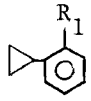
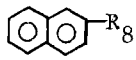
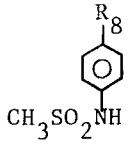
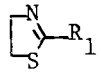
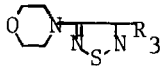
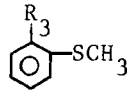
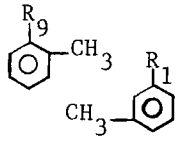
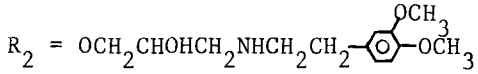
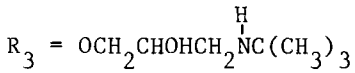
Exaprolol	MG 8823		E	-	-	+	52
Indenolol	YB-2		E				53
Iprocrolol	P-16		E	-	-		54
Labetolol[g] (Trandate)	AH 5158		M	-	-	+	55
Mepindolol (Corindolan)	LF 17895		C	-	+	+	56
Metipranolol[h] (Disorat)	VUFB 6453		M	-	-		57,58
Metoprolol (Lopressor)	H 93/26		M	+	-	+	59
Moprolol (Morolol)	SD 1601		C			+	60
Nadolol (Corgard)	SQ 11725		M	-	-	-	61
Nifenalol	INPEA		M	-	+	-	62
Oxprenolol (Trasicor)	Ba 39089		M	-	+	+	63
Pamatolol	H 104/08		C	+			64
Pargolol	Ko 1400		E	-		+	65
Penbutolol	Hoe 893d		C	-		+	66
Pindolol (Visken)	LB 46		M	-	+	+	67

Table I (cont.)

Practolol (Eraldin)	ICI 50172		MR	+	+	-	68
Procinolol	SD 212401		C	-		-	69
Pronethalol (Alderlin)	ICI 38174		W	-	-	+	70
Propranolol (Inderal)	ICI 45520		M	-	-	+	71
Sotalol (Sotacor)	MJ 1999		M	-	-	-	72
Tazolol	RS 6245		E	-	-	-	73
Timolol (Blocadren)	MK 950		M	-	-	+	74
Tiprenolol	Du 21445		E	-			75
Tolamolol	UK 6558		C	+	-	-	76
Toliprolol	Ko 592		M	-			77

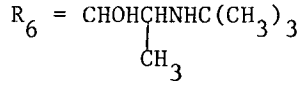
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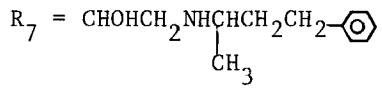
R_2 = 

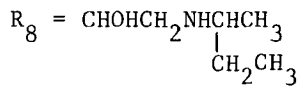
R_3 = 

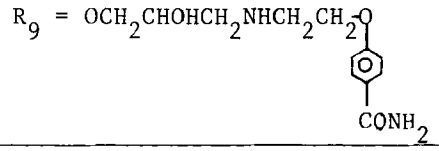
R_4 = $CHOHCH_2NHC(CH_3)_3$

R_5 = $CHOHCH_2NHCH(CH_3)_2$

R_6 = 

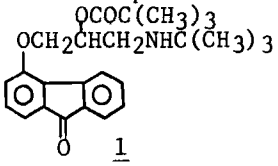
R_7 = 

R_8 = 

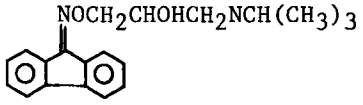
R_9 = 

[a] Status(Developmental): E, Experimental; C, Clinical; M, Marketed; MR, Marketed with restricted use; W, Withdrawn from study or use in man; [b] Cs, Cardioselective; [c] PA, partial agonist activity; [d] MSA, Membrane stabilizing activity; [e] Chemistry and/or pharmacology reference; [f] Selective for β_2-receptors; [g] α-blocking action; [h] Also known as methypranol and trimepranol.

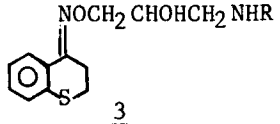
New Chemical Types - LL 21945 (1) represents a significant structural deviation from standard β-blockers.[78] The central ring structure is a 9-fluorenone. In addition, the secondary hydroxyl group of the aliphatic side chain is esterified. Presumably, the ester group is slowly hydrolyzed during biotransformation. This action results in slow release of an active metabolite which accounts for the long duration of action of LL 21945.[79] An oxime derivative of 9-fluorenone, IPS 339 (2), possesses significant β-blocking action with preferential action on β_2-receptors.[80] Oxime derivatives of thiochromanones, of the general structure 3, have also been reported to have β-blocking action.[81]



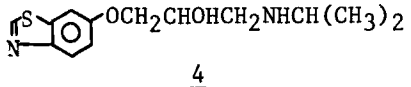
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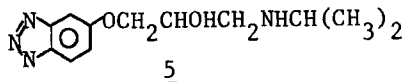
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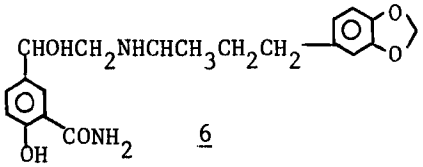
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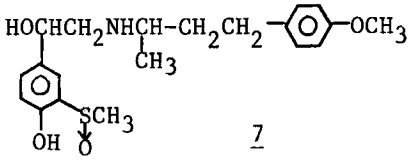
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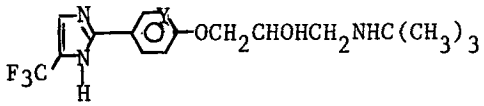
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7



(8) Y = CH
(9) Y = N

The structure of KF-577 (4) differs from the usual aromatic ring system by the incorporation of a benzthiazole ring.[82] This agent has a pharmacological profile similar to that of propranolol with the exception of a low level of cardioselectivity.[83] The related compound, 5, a benztriazole analog, has also been reported to possess significant β-blocking activity.[84]

RMI-81968 (6), a 1,3-benzydioxol-5-yl substituted alkyl amine possessing both β- and α-blocking activities, has been reported to also produce direct vasodilation.[85] A similar structure, 7, WIN 40808-7 (sulfinalol),[86] possesses vasodilator action in addition to β-receptor blocking properties.[87] Both β-blocking action and vasodilatory activity have been reported for 2-phenyl- and 2-pyridyl-4-trifluromethyl-imidazoles, 8 and 9.[88,89]

New Concepts - "Combined action" blockade of both β- and α-receptors as demonstrated with labetolol and RMI-81968 has been suggested to offer an advantage over conventional β-blockers in the treatment of hypertension.[90] Compounds possessing both β-blocking action and vasodilator activity have been developed with the primary goal of attenuating the tachycardia associated with vasodilator therapy.[88]

Diuretics have been combined with β-blockers in fixed combinations as exemplified by clopamide and pindolol (Viskaldix®) and by metoprolol

and hydrochlorothiazide (Co-Betaloc®), for the treatment of mild to moderate hypertension.[91]

A simple regimen of treatment with one-a-day medication is possible for example with a long-acting propranolol formulation.[92] A slow-release oxprenolol formulation has been reported to produce better therapeutic results than conventional formulation of several β-blockers.[93-95] The apparent advantage of these formulations is thought to be due to better patient compliance. This advantage would also be shared by β-blockers which have an intrinsically long duration of action. Nadolol given once daily has been reported to be as effective as propranolol administered four times daily in the treatment of angina pectoris.[96] Similarly, sotalol effectively controlled blood pressure in hypertensive patients on a once-daily dosage regimen.[97]

Other Clinical Indications - The list of clinical indications in which β-blockers have either been proven efficacious or are now being studied continues to grow. In addition to their now accepted efficacy in angina,[98,99] myocardial infarction,[100,101] arrhythmias[102] and hypertension,[103,104] other disease states characterized by excess sympathetic activity such as thyrotoxicosis [105] have been treated with β-blockers.

β-Blockers have also been reported to be useful drugs in treatment of migraine attacks,[106,107] anxiety,[108] and tremor.[109] β-Blockers are advocated for use in conditions such as schizophrenia and mania.[110] These agents are possibly useful in opiate and other drug withdrawal.[111] The introduction of β-blockers in ophthalmology represents an important advance in treatment of glaucoma.[112] Timolol has been studied extensively and it is reported to effectively reduce intraocular pressure in patients with open angle glaucoma or ocular hypertension.[113]

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Chapter 10. Histamine Receptors

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Introduction - The classification of histamine H_1 receptors[1] in 1966 and of histamine H_2 receptors[2] in 1972 reawakened interest in histamine; interest has intensified since the introduction of the specific H_2-receptor histamine antagonist, cimetidine (TAGAMET), in 1976 for the control of gastric acid hypersecretion and treatment of peptic ulcers. The identification of specific antagonists and selective agonists has given great impetus to pharmacological analysis of histamine actions and in the last few years there has been a rapid growth of the literature on histamine pharmacology.

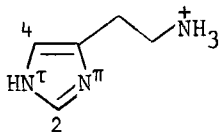
Histamine receptors were reviewed in 1973 before the advent of the H_2 antagonists;[3] later developments are described in reference 4, and the subject was mentioned briefly in these reports[5] in 1975 and 1977. The classification and distribution of histamine receptors has been discussed by Black[6] and reviewed by Chand and Eyre.[7] The Physiological Society of Philadelphia held a symposium on histamine receptors in 1977, the proceedings of which are in press.[8] A symposium was held in 1978 at the VIth International Conference on Medicinal Chemistry, Brighton, England (proceedings to be published). In addition, there have been several symposia on H_2-receptor histamine antagonists, mainly concerned with clinical aspects.[9-12]

Drugs Acting at Histamine Receptors

Agonists - The existence of two receptor populations for histamine raises the interesting question of whether the chemical mechanism of histamine interaction differs between the two receptor types. Some indications of the chemical properties which may differentiate receptor action come from studies of histamine chemistry and from structure-activity considerations of congeners.[13] Histamine in aqueous solution is a mixture of equilibrating species, viz. ionic forms, tautomers and conformers; ^{13}C nmr studies[14] confirm earlier pK_a work[15] indicating a N^{τ}-H:N^{π}-H (structures 1 and 2) tautomer ratio of approximately 4:1 for histamine monocation, and a comparable ratio for histamine base.[16] The latter result contrasts with crystal structure data[17] and molecular orbital predictions,[18-19] and may indicate an influence of solvent on tautomer stability. Recent studies of properties pertinent to consideration of ligand-receptor interactions are conformation (MO calculations[20-21] and infra-red comparison of solid state and chloroform solutions of histamine base[22]), electronic charge distribution,[23] metal complexation,[24] and phospholipid interactions.[25]

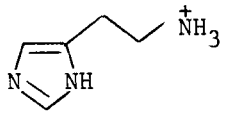
The identification of selectively acting agonists, chemically related to histamine, provides a valuable basis for studying the medicinal chemistry of histamine. Thus, 2-pyridylethylamine (3, R=H) and 2-thiazolylethylamine (4) have appreciable activity as agonists at H_1 receptors and therefore indicate that although the N^{τ}-H tautomer (1) of histamine is

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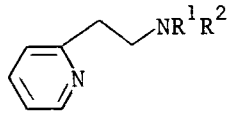
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N^{τ}-H tautomer

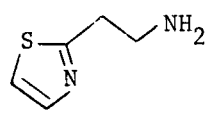


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N^{π}-H tautomer

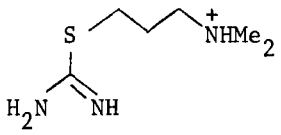


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4

likely to be an active form, tautomerism is not a pre-requisite for activity at H_1 receptors.[13] These compounds are not very active at H_2 receptors which suggests that the chemical features of a particular tautomeric structure, or tautomerism, may be important for H_2 receptor stimulation.[13] It has been suggested that the histamine N^{τ}-H tautomer may bind to the receptor via hydrogen donor and acceptor bonding and it may also function as a proton transfer agent;[13,26] such a mechanism is analogous to the function of imidazole in the histidyl residues of certain enzymes. Molecular orbital calculations provide a similar model.[19]

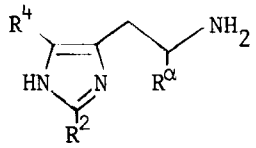


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dimaprit monocation

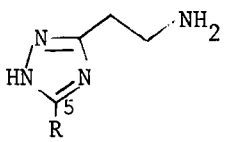
The H_2-receptor agonist, dimaprit[27] [S-(3-N,N-dimethylaminopropyl)isothiourea] (5) which lacks an imidazole ring, could nevertheless function analogously to histamine since the isothiourea group is a tautomeric amidine and may provide hydrogen donor and acceptor bonding through the amidino nitrogen atoms.[27] An alternative view regards the sulphur atom as an H-bond acceptor.[92] Isothiourea and imidazole are both planar 6Π-electron systems and in the dimaprit-histamine comparison they represent a very interesting and novel example of bio-isosterism.[27] Dimaprit is highly specific for H_2 receptors; in vitro it has, respectively, 17 and 70% of the potency of histamine on the rat uterus and guinea-pig right atrium (H_2 systems) but less than 0.0001% on the guinea-pig ileum (an H_1 system),[28] a ratio of better than 1:100,000. Dimaprit stimulates gastric acid secretion in the rat, dog and cat, although there are differences in potency. In the dog and cat the maximum secretory response to dimaprit was significantly greater than that obtained to histamine.[28,29] Dimaprit is especially useful for studying H_2-receptor mediated cardiovascular responses.[28,30] Dimaprit poses an interesting problem for conformational analysis since the higher and lower homologs are not active.[27,31]

Substituents in the heterocyclic ring may impart selectivity of action. Thus 4-methylhistamine (6b) is a selective H_2-receptor agonist[2] (some authors refer to this compound as 5-methylhistamine), 2-methylhistamine (6a) is a relatively selective H_1-receptor agonist,[2] and their N^{α}-methyl derivatives[32,33] are also correspondingly selective (numbering according to ref. 34). Presumably, the methyl group interferes with molecular function; this may occur as a simple steric inhibition of agonist-receptor 'fit' but an alternative explanation is provided by a study of the conformation of methyl derivatives which defined an essential conformation for H_1 receptor activity.[35] Various rigid analogs of histamine have been tested but none is particularly active.[36] Branching of the histamine side chain markedly reduces ability to act at either receptor.[32] Surprisingly, this does not appear to hold for 4-methylhistamine since α,4-



	R^2	R^4	R^α
a	Me	H	H
b	H	Me	H
c	H	Me	Me
d	H	Et	H
e	H	nPr	H

6



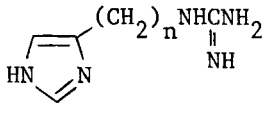
	R
a	H
b	NH_2
c	Me

7

dimethylhistamine (6c) is reported to be selective, having about 5 - 10% of the potency of histamine at H_2 receptors.[37] 4-Ethylhistamine (6d) is also a selective H_2-receptor agonist[31,38] but larger 4-alkyl groups hinder agonist function.[38] 3-(1,2,4-Triazolyl)ethylamine (7a) is an agonist at both receptors but, interestingly, a 5-amino substitutent (7b) imparts selectivity towards H_2 receptors whereas the 5-methyl derivative (7c) (structurally analogous to 2-methylhistamine) appears to be specifically active at H_1 receptors.[39]

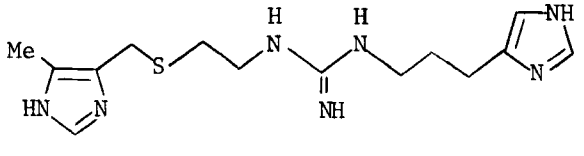
Partial Agonists - Increasing the size of side-chain N-substituents in 2-pyridylethylamine (3) reduces H_1-receptor agonist potency. When R^1 or R^2 = Et, compounds are partial H_1 agonists, but higher alkyl homologs (R^1= nPr, R^2 = H) are purely H_1 antagonists.[40] Antagonist potency is enhanced by an increase in size of the substituent. A detailed study shows that the substituent makes a major contribution to receptor affinity whereas the pyridylethyl contributes little to affinity.[40] Similar substituent effects obtain for the histamine molecule, partial agonism at H_1 receptors appearing when cyclohexyl or aryl substituents are incorporated in the 2-position.[41] Increase in size of the 4-substituent in histamine reduces H_2-receptor agonist potency and, in fact, leads to partial agonism. Thus 4-propylhistamine (6e) has only 7% of histamine's potency on the guinea-pig isolated right atrium and achieves only 50% of histamine's maximal response.[38]

There have been some interesting developments with guanidine derivatives. N^α-Guanylhistamine (8a) is a very weak partial H_2 agonist.[42a] The homolog (SK&F 91486, 8b) is a more potent partial agonist[42b,42c] but further extension of the side chain (as in the N-methyl thia-linked analog, SK&F 92408, 13a) gives an H_2 antagonist which has no agonist activity.[42d] A fascinating compound is obtained by combining the imidazolylalkyl groups (8b and 13a) into a single guanidine structure viz. SK&F 92676, impromidine, (9); this compound is a very potent selective H_2-receptor agonist, and is nearly 50 times more potent than histamine on the guinea-pig atrium preparation.[43] Impromidine is less active on the rat uterus preparation (9 times more active than histamine) and it behaves as a partial agonist,



a n=2
b n=3

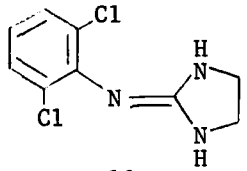
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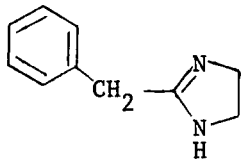
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achieving only 80% of histamine's maximal response, which indicates that the increase in activity relative to histamine is due to increased affinity for H_2 receptors rather than increased efficacy. Yet, a chemical group which contributes affinity in antagonist structures apparently converts a weak partial agonist (8b) into an agent which has increased efficacy as well as increased affinity; this result has important implications in considering drug-receptor interaction. Impromidine is a potent stimulant of gastric acid secretion and may be useful as a diagnostic agent for the estimation of the maximal secretory capacity of the stomach.[43,44]

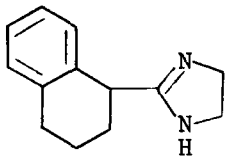
Other amidines behaving like partial agonists are clonidine (10) and tolazoline (11). Various suggestions have been made that clonidine stimulates CNS H_2 receptors,[45,46] but on peripheral tissues clonidine appears to be a very weak partial H_2 agonist[47] and the studies suggest that the action of clonidine at H_2 receptors is complex. Tolazoline has been estimated to have 3% the agonist activity of histamine on the guinea-pig isolated right atrium[48] but it behaves like a partial agonist giving a sub-maximal response.[48,49] Tolazoline may lead to H_1 and H_2 receptor



10



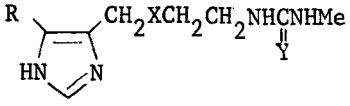
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12

responses,[50] and may act via histamine release.[51] The closely related compound, tetrahydrozoline (12), also acts as a histamine-like partial agonist[49] and this activity appears to be associated with the d-isomer[52] but not the l-isomer.

Antagonists - Recent structure-activity studies of H_1-receptor antihistamines have been concerned with stereochemical aspects,[53,54] partition characteristics,[40,55,56] pattern recognition,[57] affinity contributions to activity[40] and association phenomena.[58,59] The subject has also been reviewed recently.[60] Undoubtedly, the main focus of attention has been on the H_2-receptor histamine antagonists. Three compounds have been widely studied, viz. burimamide[2] (13b), metiamide[61] (13c) and cimetidine[62] (13d), and their pharmacological properties summarized.[63] Their discovery and development at SK&F was the culmination of a research programme initiated in 1964. The search for an antagonist and some of the medicinal chemical approaches used in the structure-activity analysis have been outlined in several articles.[42c,64,65] Concurrently, other researchers had sought



13

	R	X	Y
a	H	S	NH
b	H	CH_2	S
c	Me	S	S
d	Me	S	N.CN

antagonists using the early H_1-antihistamine 929F as a starting point; they obtained an antisecretory agent but not an H_2-receptor antagonist.[66]

In structure-activity analyses of the H_2-receptor histamine antagonists, physicochemical properties such as acidity, hydrophility, dipole moment and geometry have been emphasized. Thiourea, urea, guanidine, cyanoguanidine and nitroguanidine comprise the variable structural units of a small homogeneous group of compounds (13, Y = S,O,NH,N.CN,N.NO_2) which provide the basis for analysis of physicochemical properties in relationship to biological activity.[67,68] Thiourea, nitroguanidine and cyanoguanidine are thereby regarded as bioisosteres with respect to action at histamine H_2 receptors; the functional similarity between thiourea and cyanoguanidine is emphasized by the parallelism between variously substituted imidazoles, and also from a detailed analysis of their physicochemistry.[67] The extent of the isosterism is strikingly illustrated by the almost identical molecular arrangements found in crystal structures of metiamide[69] and cimetidine.[70] Comparison of CH_2 with thioether S linkages by crystal structure and solid-state infra-red spectra suggest that the thioether linkage may increase conformational flexibility and favor folding of the side chain; this effect may contribute to H_2-receptor antagonist activity.[69] An interesting correlation is found between crystal stability, m.p. and aqueous solubilities of these compounds.[69] Alkyl branching of the methylene adjacent to the imidazole ring lowers activity[71] but whether this is a conformational effect has not been studied.

Biological Distribution of Histamine Receptors - In the original classification of histamine receptors[1,2] H_1-receptors were defined as mediating those actions of histamine that were blocked competitively by mepyramine, eg. the action of histamine in stimulating contraction of smooth muscle from the gut and bronchi. H_2-Receptors were identified from those actions of histamine, refractory to mepyramine but blocked competitively by burimamide, viz. stimulation of rate of the spontaneously beating guinea-pig isolated right atrium (chronotropic effect), inhibition of evoked contractions of the isolated uterus from the rat, and the stimulation of gastric acid secretion. Both receptors appeared to be involved in mediating the depressor effect of histamine on blood pressure, as demonstrated in the cat.[2] There has since been a considerable pharmacological investigation of histamine actions in various systems and species.[6,7]

The discovery of H_2-receptor histamine antagonists has rekindled interest in histamine physiology. Three stimulants of gastric acid secretion, generally regarded as being of physiological importance, are histamine, gastrin and acetylcholine. The H_2-receptor histamine antagonists and, to a lesser degree, anticholinergics can inhibit acid secretion induced by all three agents *in vivo*, and various models have been proposed involving sequential hormonal actions or interacting receptors on the parietal cell.[72] To simplify the complex situation *in vivo*, studies also continue with *in vitro* preparations eg. isolated stomachs,[73] mucosal sheets,[74] or isolated parietal cells.[75]

A comprehensive review of histamine receptors in the cardiovascular system has been published.[76] Histamine lowers blood pressure by causing widespread vasodilatation in most animal species but the effects are complex to analyse. It is clear that both H_1 and H_2 receptors are involved, but their distribution and effect varies depending on the species and particular vascular bed under study. Both types of antagonist have to be administered together to fully block many of the vascular effects of

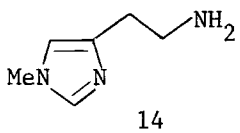
histamine.

In addition to the well known H_1-receptor effects of histamine in stimulating contraction of smooth muscle from parts of the gastro-intestinal tract, lungs and genito-urinary systems, there have been various reports of inhibitory (relaxant) actions of histamine which are apparently mediated by H_2-receptors eg. in the lower esophageal sphincter of the opossum,[77] bovine stomach,[78] chicken ileum,[79] guinea-pig gall bladder,[80] trachea and bronchus of various species[81-83] including man,[84] mouse vas deferens.[85] The distribution of receptors is species dependent and in many cases both receptor types appear to be present and causing opposing effects. This raises interesting questions about possible function and requires careful study with respect to both drug concentration and time dependency. The possibility of both types of receptor being present in the guinea-pig ileum may have a bearing on the effect of temperature in apparently altering receptor population.[82] In some circumstances, eg. cat bronchus and horse trachea, there may be actions of histamine not mediated by either H_1 or H_2 receptors.[83]

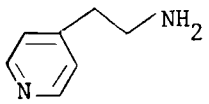
One of the fascinating developments is the discovery of histamine receptors in the immuno-regulatory system. Histamine has been shown to inhibit its own release,[119] inhibit T-lymphocyte-mediated cytolysis (indeed, T-lymphocytes may develop H_2-receptors as they mature),[86] inhibit enzyme release from leukocytes,[107] inhibit production of migration inhibitory factor,[87] modulate eosinophil migration,[88] and suppress human lymphocyte responses to mitogens.[89] H_2-Receptors appear to be involved in mast cells, basophils, T-lymphocytes and neutrophils. This array of histamine effects holds a tantalizing prospect for future drug investigation.

In recent years evidence has accumulated to suggest that histamine may act as a neurotransmitter in the brain but direct evidence of a function is still lacking. Histamine is present in the brain, its distribution is uneven and histamine sensitive neurones have been demonstrated using microelectrophoretic measurements. However, administration of histamine, selective agonists, or antagonists have not clearly indicated whether there are CNS histamine receptors resembling the peripheral receptors.[90-92] The last 2 or 3 years has seen a dramatic change, however, because investigations of cAMP accumulation in brain tissue, and direct binding studies, suggest the presence of both H_1- and H_2-receptors (see below). Furthermore a fascinating interaction with anti-depressant drugs has emerged.[113-114]

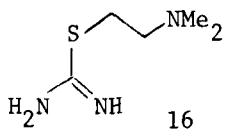
Chemical Control Substances - Pharmacological classification of histamine receptors rests on the use of the specific competitive antagonists, supplemented by studies with relatively selective agonists and, ideally, characterization requires that the agonist - antagonist interaction is shown to be competitive.[93] Where histamine is not administered but the possible involvement of endogenous histamine is being investigated, classification poses a special problem. Because drugs (whether agonist or antagonist) may cause effects other than the intended action at receptors, it may be helpful to use chemical controls i.e. compounds which match many of the chemical properties of the active drugs but lack the specific structural properties needed for effective receptor interactions, viz. N-telemethylhistamine,[2,32] (14) (cf. histamine or 2- or 4-methylhistamines, 6a, 6b) 4-pyridylethylamine,[13] (15) (cf. selective H_1 agonist 2-pyridylethyl-



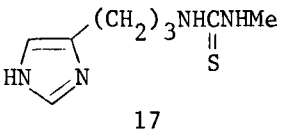
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15



16



17

amine, 3) S-(2-dimethylaminoethyl)isothiourea,[27] (16, SK&F 91487, 'nor-dimaprit') (cf. selective H_2 agonist, dimaprit, 5) N-methyl-N'-[3-(imidazol-4-yl)propyl]thiourea[65,94] (17, SK&F 91581, 'norburimamide') (cf. H_2 antagonists, burimamide 13b, or metiamide 13c).

Cyclic Nucleotide Responses - Many H_2-receptor mediated actions of histamine have been shown to be associated with increased levels of cyclic adenosine 3',5'-monophosphate (cAMP).[95] Histamine stimulated adenylate cyclase systems from gastric mucosa have been found in most species[96,97] including man[98] and results with antagonists and selective agonists indicate that this is an H_2-receptor effect. Most work on histamine and cardiac adenylate cyclase has used the guinea-pig heart[99] and demonstrated that the inotropic effect of histamine (increase in force of contraction) on the isolated heart is closely preceded by increases in cAMP levels; this appears to be H_2-receptor mediated. Studies on broken cell preparations indicate regional differences; the ventricle appears to contain an H_2-receptor histamine-sensitive adenylate cyclase but in the left atrium, where the inotropic response to histamine is an H_1-receptor system, cAMP does not seem to be involved.[100,101] In the right atrium, where the chronotropic response to histamine is mediated by H_2 receptors, it has not yet proved possible to identify an associated adenylate cyclase.[101] Histamine increases cAMP levels in the brain[102] depending on species, brain region, and method of tissue preparation eg. intact cells (brain slices) versus broken cells (washed membrane preparations),[103,104] additions of adenosine[102,105] or GTP.[106,92] The guinea-pig has been most studied and, from the use of specific antagonists[108,109,110] supplemented by selective agonists[104,106,109,110] it appears that both H_1 and H_2 receptors may mediate cAMP formation. Results have been variable and conflicting, probably due to differences in methodology. In the rat[111] and chick[112] the receptors appear to be H_2; in the rabbit,[103] H_1. D-Lysergic acid diethylamide, its 2-bromo-derivative, and many antidepressant drugs are also potent inhibitors of histamine-sensitive adenylate cyclase in homogenates of guinea-pig hippocampus or neocortex;[104,92,113,114] they also inhibit dimaprit stimulation and thus appear to act at H_2-receptors.[92,113] Histamine increases cAMP formation via H_2-receptors in various other systems, eg., segments of rat uterus,[115] canine fat cells (leading to lipolysis),[116] mouse thyroid lobes (to facilitate thyroid hormone secretion),[117] glomureli isolated from rat renal cortex,[118] human leukocytes[119] and neutrophils.[107] Histamine may stimulate formation of guanosine 3', 5'-monophosphate (cGMP) via an H_1-receptor, eg. in mouse neuroblastoma cells,[120] human and guinea-pig lung.[121]

Receptor Location and Binding Studies - Mepyramine, tritiated in the 5-position of the pyridine ring has been synthesized[122] and specific binding to homogenates from guinea-pig small intestine demonstrated.[123] Inhibition of ^{3}H-mepyramine binding by H_1-antagonists gave affinity constants in good agreement with *in vitro* activities for antagonizing histamine stimulated contraction of ileum, suggesting that the observed specific binding is to

histamine H_1-receptors. Subsequently, binding of ^{3}H-mepyramine to membrane fractions from guinea-pig or rat whole brains has also been demonstrated.[124,125] Various phenothiazine antipsychotic drugs also bind to the mepyramine site[126] in brain tissue but there are indications that ^{3}H-mepyramine binding may not represent a simple equilibrium with a single set of binding sites.[124] Binding of ^{3}H-cimetidine to homogenates of guinea-pig whole brain,[127] or with a particulate fraction of gastric mucosa from rats,[128] has been studied but this does not necessarily represent H_2-receptor binding. Binding of ^{3}H-histamine to homogenized regions of rat brain,[129] or of ^{14}C-histamine to plasma membranes from rat gastric mucosal cells,[130] has also been studied.

Histamine receptors have not so far been identified by chemical or physical means but there have been attempts[131] to isolate and purify the histamine H_1-receptor from a membrane fraction of smooth muscle of the cat small intestine. Binding of ^{3}H-histamine to this fraction appears to be to H_1-receptors, and is consistent in comparison with 2- and 4-methylhistamines.[132] Affinity labelling with the β-haloalkylamine, Dibenamine, was thwarted by lack of specificity, and a potentially more specific alkylating agent (diphenhydramine mustard) was developed.[133] However, subsequent work on the guinea-pig ileum with the corresponding aziridine suggests[134] that this compound does not alkylate the H_1 receptor. Clearly there is a great need for specific affinity labels for both H_1- and H_2-receptors.

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Section III - Chemotherapeutic Agents

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Chapter 11. Antibiotics

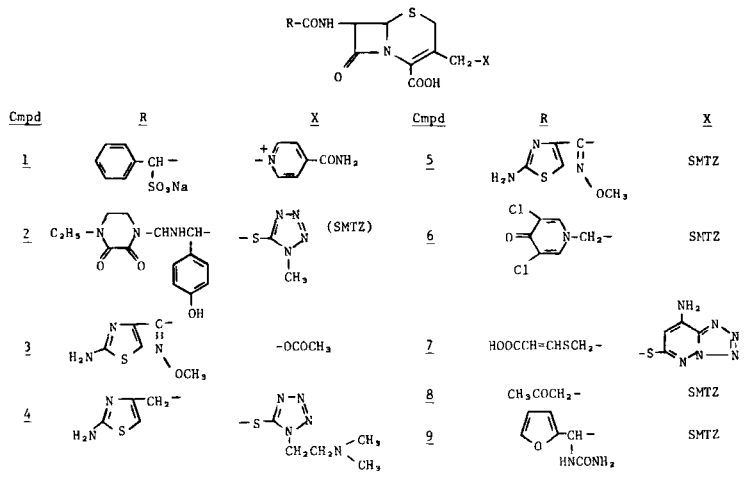
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Introduction - This chapter will be limited to antibiotics for which antibacterial activity has been demonstrated. Subjects related to biosynthesis are excluded. A variety of antibiotic topics were presented at the 18th Interscience Conference on Antimicrobial Agents and Chemotherapy, for which abstracts were published.[1] The proceedings of the previous year's 10th International Congress of Chemotherapy cover a variety of clinical topics.[2] Two books of general interest are concerned with the indexing, isolation, separation and purification of antibiotics.[3,4]

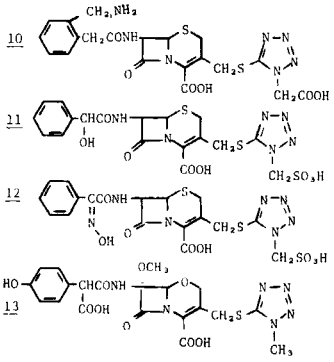
Cephalosporins - Most of the new cephalosporins have been designed to improve the shortcomings of the available products. Some under clinical development are wider spectrum agents possessing antipseudomonal activity and good β-lactamase stability. Several others show favorable pharmacokinetics and offer the potential for reduced dosage or frequency of dosing. It is worthy of note that no significant orally-active cephalosporins were reported.

Interest has continued in cephalosporins with antipseudomonal activity. Cefsulodin (SCE-129, CGP 7174, 1) shows moderate *in vitro* and *in vivo* activity against *P. aeruginosa* and gram-positive cocci with poor general activity against gram-negative organisms despite its high resistance to hydrolysis by various β-lactamases.[5-7] Its pharmacokinetics and metabolism have been studied in experimental animals.[8,9] It is well tolerated in man and achieves good serum levels, exceeding those of carbenicillin upon IM or IV administration.[10,11] An effective clinical response in 54% of 320 patients with various pseudomonal infections was reported.[12] T-1551 (2), the cephalosporin analog of piperacillin, has good *in vitro* activity against *P. aeruginosa* and a broad spectrum of gram-negative bacteria. It is stable to cephalosporinases from gram-negative bacteria, but less so to penicillinases.[13] The pharmacokinetic profile is similar to that of cefazolin; however, lower urinary excretion was observed.[14] Good clinical activity was obtained against most susceptible organisms but only 3/9 patients with pseudomonal infections responded.[14] HR756 (cefotaxime, 3) has remarkable broad spectrum *in vitro* activity.[15-21] *B. fragilis* and *Serratia* are relatively resistant,[22] as are pseudomonal strains. Resistance may be conferred by a modification of the permeability barrier.[17,23] HR756 was not hydrolyzed by Richmond type I, III, IV and V β-lactamases and was found to be an inhibitor of β-lactamases.[24] It offered good protection to mice infected with bacterial organisms.[25] Initial clinical studies[26-30] show it to be effective upon parenteral administration; however, the relatively low serum levels and half-life may limit its use in the treatment of pseudomonas infections.

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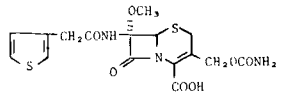


SCE-963 (4)[31] has broad activity against bacteria[31,32] Pharmacokinetics in animals show lower blood levels and urinary excretion than with cefazolin.[33,34] Preliminary clinical studies are positive to date.[35] A related compound SCE-1365 (5) is reported to possess broader *in vitro* antibacterial activity with improved β-lactamase resistance.[36] Cefazedone (6) a narrow spectrum cephalosporin,[37] gave low serum levels in man following IM or IV administration but good tissue penetration.[38] K13176 (7) demonstrated broad spectrum parenteral action in limited laboratory studies.[39] The synthesis and antimicrobial profile of a series of 7-(β-heteroacylamino)cephalosporins have been described.[40] Of these, the most active member, 8, showed moderate antibacterial activity *in vitro* and *in vivo*. Several new cephalosporins containing 2-pyridinethiol 1-oxide groupings were found to have *in vitro* antifungal activity.[41] None of these compounds protected mice infected with *C. albicans*, despite being active *in vivo* against bacterial species. Of interest are a group of 7-ureidoacetyl cephalosporins in which the L-side chain isomers were potent antibiotics and had greater potency against gram-negative bacteria than the corresponding D-side chain isomers.[42] SQ69,613 (9) was the most active member of this series.

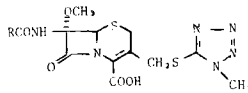


Ceforanide (BL-S786, 10) a parenteral cephalosporin with high and prolonged serum levels in man,[43,44] has broad spectrum *in vitro* activity.[45,46] Initial clinical trials using twice-a-day treatment show promise.[47-50] SK&F 75073 (11) is a new parenteral broad spectrum cephalosporin with high and prolonged serum levels in experimental animals.[51] Serum levels and half-life exceeded those of any reported cephalosporin and this is reflected in animal protection studies. Despite high binding to serum proteins, it penetrates well into normal and inflamed tissues.[52] This compound offers the potential for once-a-day treatment in man. Another long-acting parenteral cephalosporin of note is SK&F 80303 (12).[53] The semisynthetic oxacephalosporins are new chemical structures synthesized from penicillins.[54] The most promising member of this series, 6059-S (13) has broad *in vitro* and *in vivo* activity[55] and good serum levels in experimental animals following parenteral administration.[56]

Cephamycins - At present, the standard cephamycin is cefoxitin (14). A review lists the isolated natural variants as well as the various methods for the 7α-methoxylation of cephalosporins.[57] SK&F 73678 (15), CS-1170 (16) and SQ 14,359 (17) have been studied in the laboratory.[58,59] Basically these have the *in vitro* and *in vivo* properties of cefoxitin. Compound 17 is claimed to be the most potent.[59] Immunologic[60] and binding properties of 16 to penicillin-binding proteins[61] in *E. coli* and *Proteus* sp. are different from its demethoxy analog.



14



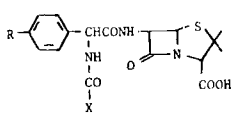
15 R = $-CH_2SCF_3$

16 R = $-CH_2SCH_2CN$

17 R = $-CH{=}NHCONH_2$

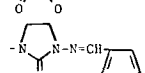


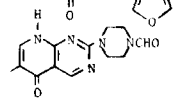
Penicillins - Interest in the penicillins is centered on broader spectrum, parenteral antispeudomonal ureido structures. Piperacillin (18) shows good *in vitro* activity, superior to that of carbenicillin against *P. aeruginosa* and other gram-negative organisms, but was hydrolyzed by many different β-lactamases.[63] Its activity against gram-positive cocci is generally comparable to carbenicillin and ticarcillin.[62-64] It is rapidly absorbed and excreted in mice and is protective against a variety of infections. Initial clinical trials suggest that it is safe and of potential value in the treatment of serious bacterial infections.[65-67] Furazlocillin (Bay k 4999, 19) has *in vitro* activity similar to piperacillin against *P. aeruginosa*.[68] It is less active than piperacillin against *B. fragilis* and shows poor resistance to hydrolysis by staphylococcal β-lactamases.[69,70] PL-385 (20) has a spectrum of *in vitro* activity similar to piperacillin but has generally better protective activity in mice.[71]



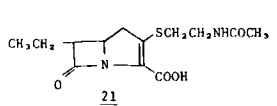
R X

18 H 

19 H 

20 OH 

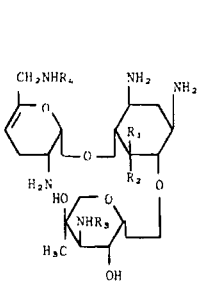
Other Inhibitors of Cell Wall Synthesis - Work continues with unusual β-lactam structures, of which thienamycin still shows high promise. The details of the structure determination of this antibiotic have been published.[72] Its total synthesis[73] and that of a number of analogs have been described, including dl-descysteaminylthienamycin[74,75] and the ring expanded analog, homothienamycin.[76] This latter compound shows greatly enhanced chemical stability over thienamycin; however, its biological activity is substantially reduced. Alaphosphin (Ro 03-7008) is a synthetic phosphonopeptide (L-alanyl-'L'-1-aminoethylphosphonic acid) which, in antagonist-free medium, is active against many gram-negative bacteria but less so against gram-positive cocci.[77,78] It protects mice infected with *E. coli* and *K. aerogenes* and is absorbed rapidly upon parenteral or oral administration to laboratory animals or man.



21 22

Inhibitors of β-lactamases - New entities have been synthesized or isolated. 6-β-Bromopenicillanic acid is an irreversible inhibitor of the β-lactamase I of *B. cereus* and *B. licheniformis*, but less so of the enzymes of *S. aureus* and *E. coli*.[79] PS-5 (21), a new natural product of a *Streptomyces* sp. A 271,

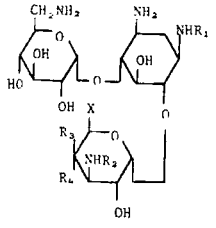
possesses good activity vs gram-positive and gram-negative organisms and also inhibits β-lactamases of various bacteria.[80] It greatly increases the activity of ampicillin and cephaloridine vs *P. vulgaris*. CP-45,899 (22) is a new, synthetic, irreversibly acting β-lactamase inhibitor with excellent solution stability.[81] It has weak antibacterial activity and potentiates the *in vitro* and *in vivo* activities of ampicillin vs β-lactamase-producing strains. Experiments continue with clavulanic acid whose β-lactamase inhibitory activity was first described in 1976. Its total synthesis[82] and that of a number of analogs[83-86] were reported. A simple and rapid color-inhibition screening method for β-lactamase inhibitors was described.[87] Beta-lactamases have been reported from yeasts[88] and *B. subtilis* ATCC 6633, a widely used assay strain.[89]



23 R_1=R_4=H, R_2=OH, R_3=CH_3

24 R_1=OH, R_2=R_3=R_4=H

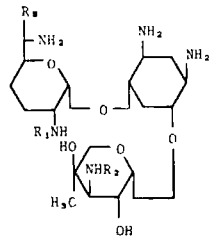
25 R_1=OH, R_2=H, R_3=CH_3, R_4=alkyl



26 R_1=COCH(OH)CH_2NH_2
R_2=R_4=CH_3, R_3=OH
X=H

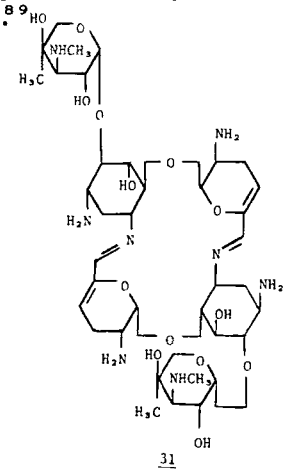
27 R_1=$CH_2CH(OH)CH_2CH_2NH_2$
R_2=R_3=H, R_4=OH
X=CH_2OH

28 R_1=R_2=X=H, R_3=OH
R_4=CH_3

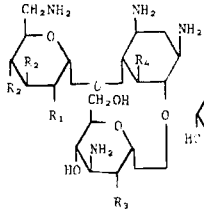


29 R_1=R_2=CH_3, R_3=H

30 R_1=H, R_2=H or alkyl, R_3=CH_3



31

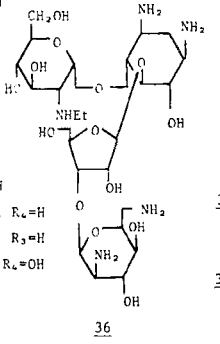


32 R_1=R_2=R_3=OH, R_4=H

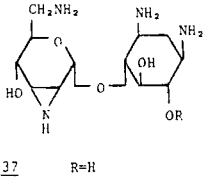
33 R_1=NH_2, R_2=R_3=OH, R_4=H

34 R_1=NH_2, R_2=R_4=OH, R_3=H

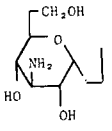
35 R_1=NH_2, R_2=R_3=H, R_4=OH



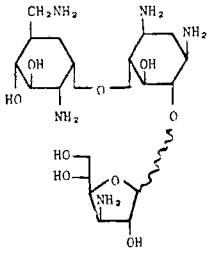
36



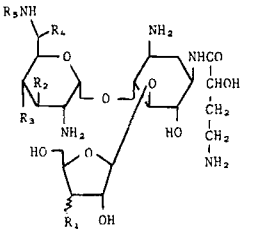
37 R=H



38 R=



39

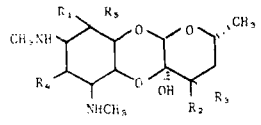


40 R_1=R_2=R_4=R_5=H, R_3=OH

41 R_1=OH, R_2=R_3=R_4=H, R_5=CH_3

42 R_1=R_2=R_3=OH, R_4=H, R_5=CH_3

43 R_1=OH, R_2=R_3=R_5=H, R_4=CH_3



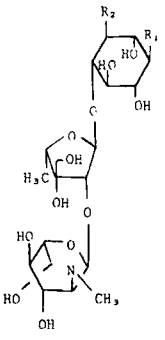
44 R_1=R_2=R_5=H, R_3=R_4=OH

45 R_1=R_5=R_4=OH, R_2=R_3=H

46 R_1=R_5=H, R_2,R_3=O, R_4=OH

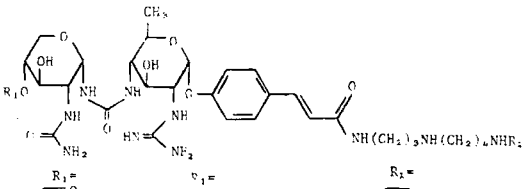
47 R_1=R_4=OH, R_2,R_3=O, R_5=H

48 R_1=R_2=R_4=H, R_3=R_5=OH



49 R_1=NH_2, R_2=NHC(=NH)NH_2

50 R_1=NHC(=NH)NH_2, R_2=H



51 (R_2=H)

52 (R_2=H)

53 (R_2=H)

54 [R_2=$CH(CH_3)_2$]

Two new mechanisms of resistance to β-lactams have been reviewed.[90] The "tolerant" *S. aureus* strains are inhibited by low penicillin concentrations, but not by high concentrations, probably due to an excess of an inhibitor of autolysin.[91] Enzyme preparations, derived from infected, human inflammatory exudate, were found to inactivate several β-lactams but not other types of antibiotics.[92,93]

Aminoglycosides - Three semisynthetic analogs warranted extensive *in vivo* and *in vitro* studies. Sch 22591 (5-episisomicin, 23) has increased potency over gentamicin against *Pseudomonas*, *Providencia* and *P. rettgeri*, as well as activity against many gentamicin resistant strains.[94] Sch 21420 (26) shows activity comparable to amikacin[95,96] as did another amikacin analog UK-18892 (27).[97,98]

New semisynthetic analogs were prepared to circumvent enzymatic inactivation in resistant strains. The 2'-N-methyl derivative of gentamicin C_{1a} (29) was almost as potent as gentamicin C_{1a} and was active against a resistant *Providencia* containing an aminoglycoside 2'-acetylating [AAC(2')] enzyme.[99] The 3"-de-N-methyl derivatives of sisomicin (24), gentamicin B (28) and gentamicin C_2 (30) and the 3"-N-alkyl analogs of gentamicin C_2 (30) displayed activities similar to their parent compounds.[100] Compound 24 was isolated as a minor component, antibiotic 66-40G, from *Micromonospora inyoensis*.[101] The unique dimeric sisomicin-like aminoglycoside 66-40C (31) was used for the synthesis of sisomicin, antibiotic G52, and a series of 6'-N-alkylated sisomicins (25) some of which displayed activity against 6'-acetylase containing strains.[102] Unlike 5-deoxyneamine, 5-deoxykanamycins A, (32)[103] and (33)[104] showed activity similar to, or weaker than their respective parents. The syntheses of 2"-deoxykanamycin B (34) and 2",3',4'-trideoxykanamycin B (35) were reported.[105] N-Alkylation of kanamycins A and B led to less active compounds.[106] 2'-N-Ethyl-paromomycin (36) was as active as its parent.[107] The 4-O-glucosamine unit of paromomycin was replaced with glucose, mannose and galactose, with only the glucosyl derivative retaining activity.[108] The 2',3'-epimino analogs of neamine (37) and kanamycin B (38) were found to be inactive.[109] The α- and β-furanosyl isomers of kanamycin B (39) had low activity,[110] as did other furanosyl neamines and paromamines.[111] Compound 40, 3',3"-dideoxy butirosin, was less active than 3'-deoxybutirosins A or B.[112] Employing "mutational biosynthesis", a neamine-negative mutant of *B. circulans* was used to prepare from various gentamines, the butirosin analogs, 41, 42, and 43, all with enhanced activity vs resistant strains.[113,114] Various axial and equatorial 3',4',5 and 6 amino-deoxy derivatives of neamine and paromamine were described.[115-118]

Attempts to overcome enzymatic adenylation of spectinomycin at its 9-position by preparing the 9-deoxy- and 9-epi- derivatives, 44, 45, 46, and 47, resulted in inactive products,[119,120] as did preparation of the 7-deoxy analog, 48.[121] Of the two monoguanidino derivatives of dihydrostreptomycin, 49 and 50, only 49 was active.[122]

A newly isolated, plasmid-determined acetyl transferase [AAC(3)-III] inactivates most aminoglycosides, including gentamicin and apramycin, but not butirosin, amikacin or fortimicin.[123] Earlier data had indicated no cross-resistance of apramycin with other aminoglycosides.[124] A broad-

spectrum enzyme, aminoglycoside nucleotidyl transferase [ANT(4',4")], capable of adenylating most aminoglycosides except gentamicin, sisomicin and apramycin, was isolated.[125] An enzyme from a resistant *S. aureus* was found to inactivate amikacin by adenylating its 4'-position and phosphorylating its 3'-position.[126] A study of the relationship of aminoglycoside accumulation and inactivation in a resistant *E. coli* was published.[127]

Among the new aminoglycoside-like structures reported are the *Nocardia*-produced novel glycocinnamoylspermidines, LL-BM123ß (51), γ_1 (52), and γ_2 (53) which are by definition not aminocyclitols since they contain no inositol moiety.[128] All three have broad-spectrum activity[129] and a semisynthetic analog, isopropyl LL-BM123γ (54) showed *in vitro* and *in vivo* activity equal to or greater than gentamicin.[130] Structures of several minor, less active, nebramycin factors have been reported.[131] Syntheses of dihydrostreptomycin,[132] sorbistin A_1,[133] and some sorbistin analogs,[134] as well as a new procedure for the preparation of 3'deoxy-α-glycosides[135] were published. The novel use of copper complexation to selectively acylate kanamycin A, the precursor to amikacin, was studied.[136] The 13_C NMR spectrum of amikacin and its isomers was reported.[137]

A 2-deoxystreptamine (2-DOS) requiring idiotrophic mutant was used to determine the presence of 2-DOS in unknown antibiotic hydrolyzates.[138] A *B. circulans* mutant was isolated which produced ribostamycin free of xylostasin.[139] A rapid luciferase-based assay of gentamicin was reported.[140] Labeled sisomicin was found to be taken up into bacterial cells at a higher rate than gentamicin.[141] The binding of aminoglycosides to acidic mucopolysaccharides was studied.[142]

Macrolides - A previously-reported basic 16-membered macrolide antibiotic, de-epoxy-rosamicin (M-4365 G2), is at least as active as erythromycin and josamycin.[143] Unlike erythromycin, it has *in vitro* activity against a host of gram-negative bacilli, including indole-positive *Proteus* spp. and mycoplasmas. It protects mice infected with *S. aureus*.[143] Deltamycins are produced by *Streptomyces halstedii* subsp. *deltae*.[144] Deltamycin A4, the most active component, was identified as carbomycin A. Demycarosylturimycin H is less active than turimycin and erythromycin vs *B. subtilis*.[145] Inhibition of polypeptide and ribosomal peptidyl-transferase synthesis by demycarosylturimycin is associated with acetylation of the 4"-position. Several diastereomeric 10,11-epoxyerythromycin B and 10-epi-erythromycin B's were synthesized with all being less active *in vitro* then erythromycin A or B.[146] A structural revision of the megalomycins by 13_C NMR and X-ray crystallography was reported.[147] A conformational study of oleandomycin and its derivatives was published.[148] Using a blocked mutant of the erythromycin-producing *Streptomyces erythreus*, biotransformation of lankamycin, darcanolide and 11-acetyllankolide was performed, but the products showed poor activity.[149]

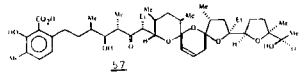
Peptides - The structure of the cyclopeptide globomycin was described.[150] This spheroplast-forming antibiotic with mainly gram-negative activity inhibits the final steps of outer membrane lipoprotein synthesis.[151] Teichomycins A_1 and A_2, phosphorous- and chlorine-containing glycopeptides produced by an *Actinoplanes* sp., are cell wall active antibiotics with mainly gram-positive activity.[152] Structural work on the thiostrepton-type

peptides multhiomycin,[153] siomycin-A,[154] micrococcins P_1 and P_2[155] and the thiopeptins,[156] was described. The structure of bleomycin was revised, replacing the β-lactam with an open carboxamide.[157] Some analogs of gramicidin S were prepared.[158,159] Studies of the solution conformation of echinomycin[160] and the effects of bicyclomycin[161] and polymyxin B[162] on bacterial membranes were published.

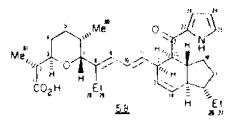
Ionophores - Several new polyether structures were published, with most bearing a close structural resemblance to earlier reported compounds.[163-165] The structures for the lasalocid-like aromatic ionophores noboritamycins A (55) and B (56), isolated from *Streptomyces noboritoensis*[166] and CP-44,161 (57) produced by *Dactylosporangium salmoneum*,[167] were reported as was that of another nitrogen containing nonpolyether ionophore, X-14547A (58) isolated from *Streptomyces antibioticus*.[168] An organic salt of an optically active amine was used in the X-ray determination of 58 to assign absolute configuration. The structures of two minor boron-containing aplasmomycin components were reported.[169] The isolation and properties of ionomycin, a calcium specific ionophore produced by *Streptomyces conglobatus*, were described,[170] as were the details the isolation and characterization of narisin[171] and corriomycin.[172] The complete assignment of the ^{13}C NMR spectrum of lasalocid was given.[173] The 300 MH_z-NMR spectrum of septamycin was used to study the solution conformations of the antibiotic and its sodium salt.[174] The effects of various ionophores on monovalent cation transport[175] and divalent cation dislocation in mitochondria[176] were investigated.



55 R=Me
56 R=Et



57



58

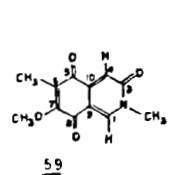
Miscellaneous Antibiotics - Other primarily antibacterial antibiotics reported in 1978 are listed in Table 1. Structural work was also published for the following previously reported compounds: mimosamycin (59)[177] griseorhodin C (60)[178] ristomycin A,[179] striatin A (61),[180] isochelocardin (62),[181] pseudomonic acid A (63),[182] and rifamycin R.[183] Marine sources have been used to obtain the new antimicrobial metabolites 64[184] and 65.[185] Syntheses of racemic negamycin and its analogs were reported.[186,187]

Table 1 New Antibiotic Entities

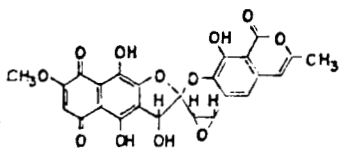
Antibiotic	Producing Organism	Activity*	Structure	Reference
A32390A	*Pyrenochaeta sp.*	G+,AF	66	195
AA-57	*Streptomyces sp.*	G+,G-	67	196
Aurantinin	*Bacillus aurantinus*	G+, Anaerob	Polyene	197
Enaminomycin A	*Streptomyces baarmensis*	G+,G-,AT,AF	68	198
Enaminomycins B,C	*Streptomyces baarmensis*	G+,G-	69,70	198
Epicorazines A,B	*Epicoccum nigrum*	G+	71,72	199,200
G1499-2	*Cytophaga johnsonii*	G+	73	201
G2201-C	*Streptomyces cattleya*	G+,G-	74	202
Rubradirin B	*Streptomyces achromogenes*	G+	75	203,204
Setomimycin	*Streptomyces pseudovenezuelae*	G+,AT	-	205

*G+,G- (gram-positive, -negative); AF (antifungal); AT (antitumor)

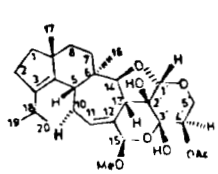
In vitro and *in vivo* data on a tolypomycin Y derivative[188] with improved activity as well as the anaerobic activity of tiamulin,[189] were published. The total syntheses of (-) vermiculine,[190] nocardicin A,[191] malonomicin,[192] capreomycin,[193] and erythronolide B[194] were reported.



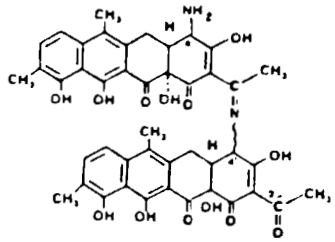
59



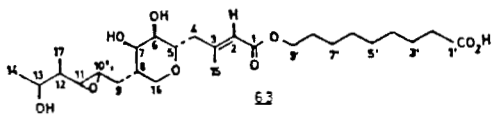
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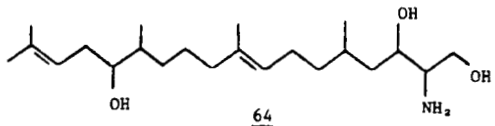
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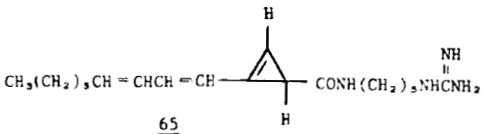
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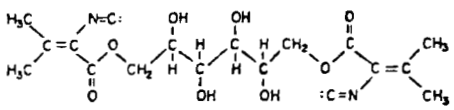
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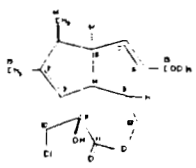
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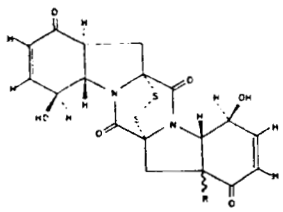
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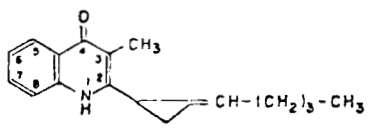


67

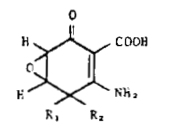


71 R= ----H

72 R= ▬ H



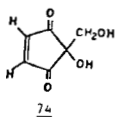
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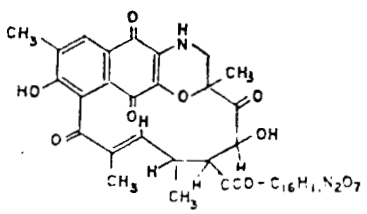
68 R_1, R_2=O

69 R_1=OH, R_2=CH_2COCH_3

70 R_1=OH, R_2=H



74



75

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Chapter 12. Chemotherapy of Sexually Transmitted Infections

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This chapter is a review of the antimicrobial susceptibilities of the causative organisms and the treatment of the sexually transmitted infections most prevalent in North America and Western Europe, gonorrhea, nongonococcal urethritis, genital herpes virus infections, and trichomoniasis. Because of their relative rarity or because no important advances in therapy have been made in the past decade, syphilis, lymphogranuloma venereum, chancroid, and granuloma inguinale are not considered.

Gonorrhea

Penicillins - For a 30-year period ending in the early 1970s, there was a gradual increase in the prevalence of strains of Neisseria gonorrhoeae with low level resistance to the penicillins, with consequent periodic increases in the minimal doses necessary for adequate treatment. Prior to 1955, 99.4% of N. gonorrhoeae isolates in the United States had minimum inhibitory concentrations (MIC) of penicillin G of $\leq$0.03 mcg/ml,[1] and aqueous procaine penicillin G (APPG) 1.2 million U intramuscularly (IM) in a single dose cured $\geq$95% of patients with uncomplicated anogenital infection. By 1975, only 31.0% of isolates had MICs $\leq$0.03 mcg/ml,[2] 17.4% had MICs $\geq$0.5 mcg/ml,[2] and APPG 4.8 million U IM, with probenecid 1.0 gm orally, was required to cure 96.8% of patients with uncomplicated infection.[3] In the past five years, susceptibility to the penicillins has remained stable,[2] probably because the recommended doses are now sufficiently high to prevent further selection of more resistant strains.[4] Ampicillin[2] and amoxicillin[5] are effective alternatives to penicillin G. Many other penicillins (talampicillin,[6] pivampicillin,[7] carbenicillin,[8] bacampicillin,[9] and others) are active in vitro and have been reasonably successful in limited clinical trials, but offer no important advantages over penicillin G, ampicillin, or amoxicillin. Penicillin V and the penicillinase-resistant penicillins are less active against N. gonorrhoeae,[10] and their use has been associated with unacceptable clinical results. The U.S. Public Health Service Center for Disease Control (CDC) recommends[11] APPG 4.8 million U IM, ampicillin 3.5 gm orally in a single dose, or amoxicillin 3.0 gm orally in a single dose for uncomplicated anogenital infection; each is given with 1.0 gm of probenecid orally. Pharyngeal gonococcal infection responds well to APPG-probenecid,[5] but less reliably to ampicillin-probenecid or amoxicillin-probenecid in single doses.[5,12] Ampicillin-probenecid is associated with slightly higher failure rates compared to APPG-probenecid in anorectal infection,[13] especially in homosexual men. Single dose therapy with any of the penicillins is inadequate for ascending genital infection in women (pelvic inflammatory disease); this complication is usually treated with 7-10 day courses of parenterally administered penicillin G and/or oral ampicillin.[14] The strains of N. gonorrhoeae that cause over 90% of cases of hematogenously disseminated gonococcal infection have retained susceptibility to $\leq$0.06 mcg/ml penicillin G,[15] making this syndrome unique among bacteremic diseases in

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that short (3-day) courses of intravenous (IV) penicillin G[16] or oral therapy alone (ampicillin 2 gm daily in divided doses for 7-10 days)[17] are appropriate and uniformly effective, if endocarditis or meningitis is not present.

In 1976, Falkow and his colleagues[18] predicted the appearance of plasmid-mediated beta-lactamase production by N. gonorrhoeae, a prediction that was soon realized.[19,20] Fear that penicillinase-producing N. gonorrhoeae (PPNG) would become a major public health problem has stimulated a search for additional alternatives to the penicillins. Although PPNG accounts for up to 25% of gonococcal isolates in some areas (notably the Philippines, Southeast Asia, and parts of Africa), they remain rare in most other areas,[20] including North America and most areas of Europe.

Other Beta-lactam Antibiotics - Several newer cephalosporins and cephamycins have been tested clinically in gonorrhea. Cefazolin, cefoxitin, cefuroxime, cefamandole, and others are effective in vitro,[10,21] but have given variable results in clinical trials.[22-24] Cefuroxime is the most effective, with cure rates in uncomplicated infection of 98-100% when 1.0-1.5 gm is given IM with probenecid 1.0 gm orally, and is effective against PPNG.[25]

Tetracyclines - Susceptibility of N. gonorrhoeae to the tetracyclines is linked to low level penicillin resistance. Gradually increasing resistance to the tetracyclines occurred through 1972, but since then there has been little change, with 86.0% of strains currently having tetracycline HCl MICs $\leq$1.0 mcg/ml and 14.0% having MICs $\geq$2.0 mcg/ml.[2] The tetracyclines are not clinically effective in single doses.[26] The regimen recommended in 1974 by the CDC,[27] tetracycline HCl 1.5 gm orally as a loading dose, followed by 0.5 gm orally four times daily for four days (total 9.5 gm), results in a cure rate of 96.2% in uncomplicated anogenital infection.[3] The 1979 recommendations[11] have eliminated the loading dose, with consequent reduction of nausea and vomiting that tended to limit patient compliance, and extended the duration of treatment to five days; cure rates with this regimen are comparable.[28] Other tetracyclines, including doxycycline,[29] minocycline,[30] and others, also are effective. PPNG are often highly resistant to the tetracyclines, with 39% and 21% of isolates having tetracycline HCl MICs $\geq$2.0 mcg/ml and $\geq$4.0 mcg/ml, respectively.[20]

Aminocyclitols - Spectinomycin HCl in a single dose of 2.0 gm IM cures 94.8% of patients with uncomplicated anogenital infection.[3,31] MICs are virtually always $\leq$32 mcg/ml[2,31] and have not changed appreciably since introduction of this drug for clinical use in 1971. Resistance of N. gonorrhoeae to spectinomycin is genetically independent of penicillin and tetracycline resistance,[32] and spectinomycin is the recommended treatment for gonorrhea that has not responded to a penicillin or tetracycline,[11] as well as for patients with allergy or idiosyncratic intolerance to these agents. It is also the agent of choice for infections caused by PPNG.[20] Resistance to spectinomycin is rare;[2,31,33] however, absolute resistance can be induced in a single step by serial passage in media containing subinhibitory concentrations of the drug,[34] and it has been recommended that its use be limited to the above indications, in the hope that induction of a resistant population of gonococci can be delayed or avoided.[33,34] Although originally effective, streptomycin is no longer useful for gonorrhea, due to a high level of resistance.[10] Gentamicin, kanamycin, tobramycin, amikacin,

and other aminocyclitols are effective in vitro,[10] and some have been used successfully in limited clinical trials,[35,36] but none has important advantages.

Miscellaneous Agents - Originally susceptible, N. gonorrhoeae is now resistant to the sulfonamides (sulfamethoxazole MIC ≥25 mcg/ml in 40% of isolates),[10] and is relatively resistant to trimethoprim (MIC ≥25 mcg/ml in 36% of isolates),[10] but synergism is present, and treatment with sulfamethoxazole and trimethoprim has resulted in cure rates of ≥94% when given in various multiple-dose regimens for at least two days.[37,38] This combination is also effective against PPNG.[20] Erythromycin was recommended by the CDC through 1974[27] as an alternative to the penicillins during pregnancy, but recently has been found to result in a failure rate of 24% when a total of 9.0 gm was given orally over four days,[39] and erythromycin is no longer recommended.[11] Rifampin has been used successfully,[40] but is not recommended, largely for ecological reasons and the likelihood of induction of resistance. Chloramphenicol is effective,[10] but is inappropriate because of potential serious toxicity.

Nongonococcal Urethritis

The nongonococcal urethritis (NGU) syndromes are sexually transmitted infections that are as frequent as gonococcal urethritis in the United States and Western Europe.[41] Approximately 40% of NGU is due to Chlamydia trachomatis.[42-44] The etiologies of the remainder are uncertain; Ureaplasma urealyticum ("T-strain mycoplasma") may be an important cause of most of the remainder,[42,44] although conflicting data exist.[43] Uncontrolled studies recently suggested a role for Corynebacterium genitalium type 1.[45] The same strains of C. trachomatis that cause NGU cause most acute epididymitis in men under age 35,[46] symptomatic cervicitis,[43] neonatal inclusion conjunctivitis,[47] and infantile nasopharyngitis and pneumonia,[48] and have been implicated in nongonococcal pelvic inflammatory disease.[49] Other serotypes cause endemic trachoma and lymphogranuloma venereum. U. urealyticum infection has not been proven as a cause of syndromes other than NGU, but has been statistically linked with prematurity and other puerperal complications.[50]

Tetracyclines - The tetracyclines are the agents of choice for the treatment of NGU.[41,51] In vitro, minocycline (MIC and MBC ≤0.03 mcg/ml)[52] and doxycycline (MIC and MBC ≤0.03 mcg/ml)[52] are more active aganist C. trachomatis than is tetracycline HCl (MIC ≤0.06 and MBC ≤0.125 mcg/ml)[52] and other tetracyclines.[53,54] U. urealyticum is most susceptible to minocycline and doxycycline, 85% of strains having MICs to both agents of ≤1.0 mcg/ml.[55] When continued for ≥7 days, several different regimens of tetracyclines have been equally successful in inducing clinical responses in NGU, including tetracycline HCl 0.5 gm four times daily[43,51] and minocycline 100-200 mgm daily;[56,57] experience with doxycycline is limited.[58] The rates of persistence or relapse within six weeks of treatment have been ≤17% for chlamydial NGU and 40-50% for nonchlamydial NGU,[57] and NGU associated with neither C. trachomatis nor U. urealyticum may have still higher rates of treatment failure.[44] The optimal duration of tetracycline therapy is debated; some studies suggest lower relapse or recurrence rates when therapy is prolonged to two or three weeks,[57,59,60] but more carefully controlled studies have shown no additional benefit when treatment was ex-

tended beyond 7 days.[44,61,62] Moreover, symptoms of NGU usually subside within the first 5-7 days of therapy,[44,51] after which patient compliance is often uncertain.

Other Agents - Erythromycin in a dose of 0.5 gm four times daily for ≥7 days is usually given for NGU when tetracycline is contraindicated. Cure rates and relapse rates are comparable to those seen with the tetracyclines,[44] but the total experience is much less than with the tetracyclines and there are no studies correlating success rates with C. trachomatis or U. urealyticum isolation. C. trachomatis is generally susceptible (MIC ≤0.5 mcg/ml).[52,54] U. urealyticum has been reported to be uniformly susceptible to erythromycin,[62a] but more recent studies[55] have shown 96% of strains to have MICs >3.0 mcg/ml. Nevertheless, erythromycin usually eradicates urethral colonization with U. urealyticum.[62b] As with tetracyclines, there are no controlled studies demonstrating additional therapeutic benefit beyond seven days treatment.

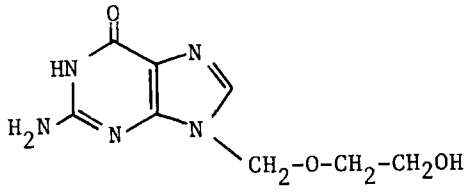
Aminocyclitols in general, and spectinomycin in particular, are effective in no more than half the patients with NGU,[42] an observation that correlates with the fact that U. urealyticum is susceptible[63] but C. trachomatis is not.[52,53] Conversely, sulfonamide therapy is successful in chlamydial NGU, but not in NGU associated with U. urealyticum. The differential response to sulfisoxazole or spectinomycin has been used to differentiate chlamydial from ureaplasmal NGU,[42] and a combination of these two agents deserves a clincial trial as an alternative to the tetracyclines.[44] C. trachomatis and U. urealyticum are individually susceptible to several other antimicrobial agents,[52-55] but all are clinically ineffective or have not been subjected to controlled studies.

Genital Herpes Virus Infections

Sexually transmitted infections with Herpes simplex (Herpesvirus hominis) type 2 is epidemic in Europe and North America. The importance of this infection is compounded by its recurrent nature, its probable etiologic association with squamous cell carcinoma of the cervix, its association with infrequent but devastating neonatal infections, and the lack of effective prophylaxis or therapy. Despite initial optimistic reports, all regimens that have been subjected to rigorously controlled clinical trials have demonstrated no benefit, either with regard to speed of healing or frequency of recurrences.[64] Immunization with smallpox vaccine, an ill-defined herpes vaccine, bacillus Calmette-Guerin (BCG),[65] and other agents is clearly ineffective.[64] Levamisole, an antihelminthic agent causing nonspecific enhancement of cell mediated immunity, is without benefit.[66]

Various topically applied agents that have been ineffective in properly controlled studies include diethyl ether,[67] cytosine arabinoside,[68] adenine arabinoside,[69] and idoxuridine,[70] although the latter agent in dimethylsulfoxide solution may hold some promise;[71] further trials are underway. Photodynamic inactivation of Herpes simplex by exposure in vitro to light in the presence of various heterocyclic dyes led to several clinical trials of exposure of genital lesions to light after application of neutral red O. Early reports were optimistic, but this treatment is clearly not beneficial,[72,73] and theoretically could enhance herpes-induced carcinogenesis.[74]

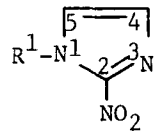
Several newer agents with antiviral activity are in various stages of testing in animal models and in clincial trials, including interferon,[75] various interferon inducers,[75] azauridine, arabinosyl hypoxanthine, trifluorothymidine, phosphonoacetic acid, and others.[64] Most promising, however, may be acycloguanosine (9-(2-hydroxyethoxymethyl)guanine) and its triphosphate,[76,77] which act by selectively inhibiting thymidine kinase to a greater degree in herpes-infected than in noninfected mammalian cells, potentially allowing for heretofore unrealized therapeutic ratios.



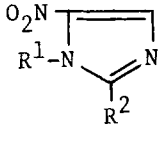
Acycloguanosine

Trichomoniasis

Vaginitis due to the parasite Trichomonas vaginalis has been successfully treated for more than 15 years with metronidazole in multiple dose regimens totalling 5-20 gm over 5-10 days, with cure rates of 90-95% when male consorts were treated simultaneously.[78] Virtually all strains of T. vaginalis are inhibited by metronidazole $\leq$3.2 mcg/ml.[79] Studies to determine the minimum effective dose have been stimulated by data suggesting significant carcinogenicity and mutagenicity of metronidazole,[80] and because of the desirability of single-dose therapy in venereal disease clinic populations. The most frequently recommended therapy at the present time is 2.0 gm orally in a single dose, with cure rates equivalent to those with multiple dose regimens.[81,82] Other nitroimidazoles, such as misonidazole (I), nimorazole, tinidazole, ornidazole, secnidazole, and carnidazole represent minor modifications, affecting pharmacokinetics rather than trichomonicidal activity, which is a function of electron transfer involving the nitro group of the imidazole ring.[83] MICs for T. vaginalis to all of these agents vary between 0.12 and 6.0 mcg/ml,[83] and all appear to be equally effective clinically. The mutagenicity and carcinogenicity of the nitroimidazoles are probably also a function of the imidazole nucleus,[80] and it is not likely that any of these agents will circumvent the question of long term safety. Unfortunately, no other drugs of comparable efficacy are available.



(I)



(II)

R^1=-CH_2CH_2OH ; R^2=CH_3 metronidazole

R^1=-CH_2CH_2-N⟨O⟩ ; R^2=H nimorazole

R^1=-$CH_2CH_2 \cdot SO_2 \cdot CH_2CH_3$; R^2=CH_3 tinidazole

R^1=-$CH_2 \cdot CH(OH) \cdot CH_2Cl$; R^2=CH_3 ornidazole

R^1=-$CH_2 \cdot CH(OH) \cdot CH_3$; R^2=CH_3 secnidazole

R^1=-$CH_2CH_2NH\text{-}CS\text{-}OCH_3$ R^2=CH_3 carnidazole

R^1=-$CH_2 \cdot CH(OH) \cdot CH_2 \cdot OCH_3$

misonidazole

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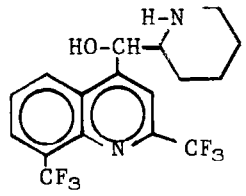
Chapter 13. Antiparasitic Agents

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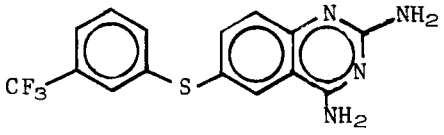
PROTOZOAL DISEASES

General - An extremely useful introductory text on the biochemistry of parasitic protozoa containing literature through 1976 was published recently.[1]

Malaria - This is not, as some would have had us believe a few years back, a rare human affliction. Its incidence is on the rise[2] and the need for improved treatment means is clearly evident from the horrifying statistic that one million children are said to die each year in Africa from malaria.[3] Resistance, the decreasing sensitivity of the malaria parasite to existing drugs, is a major problem.[4] Mefloquine (1), one of the most significant achievements in malaria chemotherapy in recent years, is effective against laboratory strains resistant to primaquine, cycloguanil, pyrimethamine and sulfaphenazole[5-8]; however, concern has been voiced for care in its application to avoid development of resistance through indiscriminant use.[9]



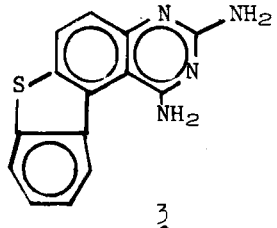
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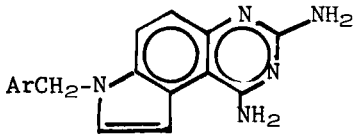
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Must reading are the papers of L. H. Schmidt summarizing his development of the owl monkey model for the evaluation of potential drugs against Plasmodium falciparum and P. vivax infections.[10-12]

Studies have appeared on a promising pyridinemethanol structurally related to 1, which is active in primates against drug-resistant strains.[13-15] Complete details on the highly potent diaminoquinazoline folate antagonists such as 2 have appeared.[16,17] The related pteridines showed lower activity against experimental infections.[18,19] Although the planar [1]benzothieno-[3,2-f]quinazoline-1,3-diamine analog 3 was devoid of antimalarial activity,[20] recent publications indicate substantial activity for the 1,3-diaminopyrrolo-(3,2-f)quinazolines 4.[21,22]



3



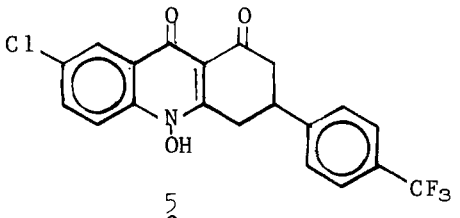
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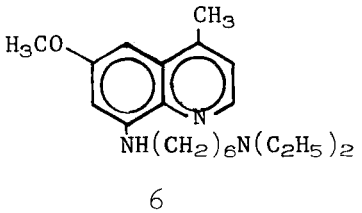
A novel structural type (5) was reported to have potent activity against resistant malaria strains.[23] Minor modifications, including removal of the N-OH substituent eliminated activity.[24] Limited clinical pharmacology, biochemistry and mode of action studies are available in the entire parasitology area. Two recent papers report the pharmacokinetics of amodiaquine, chlorguanide, chloroquine, pyrimethamine, quinine and sulfadoxine.[25,26]

Leishmaniasis - This disease, which gives every indication of dramatically increasing incidence, and is probably second in importance only to malaria of the diseases caused by protozoal parasites, is in desperate need of treatment advances. What promises to be a very exciting technological breakthrough has recently been described. The concept that drugs incorporated into liposomes could be carried more efficiently directly to lysosomes in the reticuloendothelial system in which the Leishmania parasites reside predominantly has been successfully utilized by three groups. A dose of 1.0 mg Sb/kg of Pentostam incorporated into liposomes was more than 200 times as active as the free drug against L. donovani in mice.[27] Meglumine antimoniate (Glucantime®) incorporated into liposomes was more than 300 times as effective as the unformulated drug against leishmanial infections in hamsters.[28] Substantial improvement in efficacy was also shown with a liposome preparation of antimony potassium tartrate against L. donovani infections in mice.[29]

The test system used by the Walter Reed Army Institute of Research for screening compounds against leishmania infections in golden hamsters has been described, and data were presented on a variety of 6,7-[30] and 8-aminoquinolines.[31] The most active analog, 6, was more than 700 times as active as the meglumine antimoniate (Glucantime®) standard.



5



6

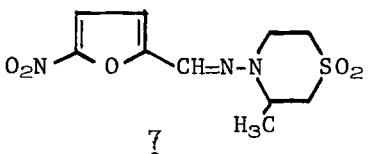
Trypanosomiasis - Little or no significant advances have occurred in alleviating either the human (American - T. cruzi; African - T. gambiense, T. rhodesiense) or the veterinary (T. brucei, T. vivax, T. congolense, T. evansi, T. equinum, T. equiperdum) forms of the disease. The veterinary forms pose a particularly serious economic problem on the African continent. As an example, trypanosomiasis has been indicted as one of the principal factors restricting growth of the livestock industry in Nigeria.[32] Surveys of the biochemistry and chemotherapy of trypanosome infections[33] and trypanocidal activity among antitumor antibiotics and other metabolic inhibitors[34] have appeared.

Human Infections: Papers included in an international colloquium on human African trypanosomiases held in 1976 have been published,[35] as have the proceedings of an international symposium on Chagas' disease.[36]

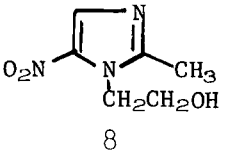
Two screens have been described using T. cruzi in the mouse.[37,38] Activity was seen with 8-aminoquinolines, a 7-aminoquinoline, a nitrofuran, p-methylbenzyltriphenylphosphonium chloride, bisquinaldines, arsenobenzenes and 2-nitroimidazoles. Nitroheterocycles have offered the most fertile ground for activity against T. cruzi. Both 2- and 5-nitroimidazole derivatives are of interest against T. cruzi[42-44] though there appears to be a distinct difference in their mode of action.[42] However, even such highly regarded compounds as nifurtimox (7) require prolonged treatment which, moreover, is not completely effective.[39-41]

Veterinary Infections: Despite previous negative results against T. brucei,[45] effective treatment of T. vivax infections was apparently achieved with salicylhydroxamic acid.[46,47]

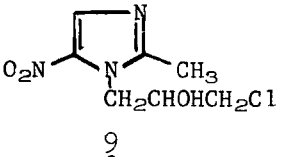
Trichomoniasis - Most of the recent literature is concerned with studies which attempt to provide sufficient data to allow a choice among several competing nitroimidazoles. Metronidazole (8), the leader in the field, continues to be explored.[48,49] A clinical isolate has been reported resistant to metronidazole,[50] and other 5-nitroimidazoles were similarly ineffective. An interesting series of articles deals with the prophylactic and therapeutic use of metronidazole for a variety of surgical indications.[51] Comparisons of metronidazole with panidazole,[52] secnidazole,[53] and ornidazole,[54] (9) have appeared. The latter is of interest because it demonstrated no effect on mammalian chromosomes although it was mutagenic in various bacterial strains. Moreover, no teratogenicity or carcinogenicity was observed in laboratory studies, and in man no evidence of alcohol intolerance was noted.[54] High activity was demonstrated not only in human trichomoniasis, but in amoebiasis and giardiasis.[54] Tinidazole (10) has been compared favorably with metronidazole.[55]



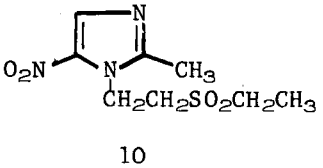
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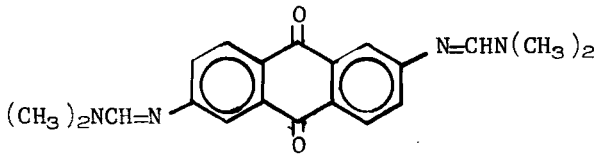
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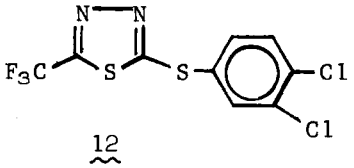
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Much less common is activity found in structures other than of the nitroheterocycle type. Activity was reported recently for the anthraquinonebisamidines 11[58] and thiadiazole 12.[59]

Amoebiasis - Little new has appeared in this area during the past two years. Several reviews, essentially clinical, have been published.[60-62] Efforts continue primarily with other nitroimidazoles to achieve superiority to metronidazole. Tinidazole (10) is receiving substantial favorable attention.[63-68] Novel bis-amidines of 2,6-diaminoanthraquinone related to 11 have shown activity comparable to the activity of metronidazole[69,70] against E. histolytica cecal infections in rats and hepatic infections in hamsters. The compounds were nonmutagenic in the Ames and dominant lethal tests.

Giardiasis - The infection of the gastrointestinal tract of man by Giardia lamblia known as giardiasis or lambliasis appears either to be increasing in prevalence or is being recognized more readily. Metronidazole and quinacrine are probably used most commonly[71] for treatment, however more effective, better tolerated therapy would be desirable. Tinidazole (10) is receiving increasingly favorable reports;[72-75] it is, however, not approved in the United States.

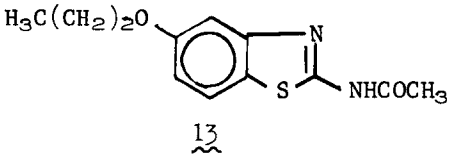
Toxoplasmosis - The obligate intracellular parasite Toxoplasma gondii is responsible for similar, although variable levels of, disease response in a wide variety of hosts including man. The disease state may not be recognized until some vital organ is compromised, and usually only the acute form responds to medication since the encysted parasites are apparently resistant to drugs which combat the trophozoites.[76] The organism is thought to be closely related to the Coccidia, and most useful agents and on-going efforts seem to center on those structural classes which inhibit the folic acid cycle. Thus, a sulfamethoxydiazine-pyrimethamine combination has been recommended for treatment of the acute disease in pregnancy.[77] Studies with 2-sulfamoyldiaminodiphenyl sulfone continue to appear but the clinical significance of this agent is not clear.[78,79] An early report of the therapeutic possibilities of chemical immunomodulation of the cellular immune response in toxoplasmosis utilizing levamisole is of interest.[80]

Coccidiosis - Control of this parasite, particularly in the poultry industry, is a problem of enormous commercial significance. The tremendous ability of the parasite to develop resistance to most known structural types[81-84] provides the major impetus for further research. Drug treatment of poultry, cattle, sheep, goats, pigs, dogs and rabbits has been reviewed.[85] Of interest is the report that no resistant isolates of Eimeria tenella were found in litter samples from broiler production flocks being treated with monensin.[86] Arprinocid [9-(2-chloro-6-fluorobenzyl)adenine] reported as a new agent last year, received a favorable report for treatment of broilers.[87] A variety of structural types such as ethanolamines,[88] pyridine-3-sulfonamide-1-oxides,[89] N-phenylpyridonehydrazones[90] and riboflavin analogs[91] have been reported to have anticoccidial activity. It is not known, however, if any of these will have any practical clinical utility.

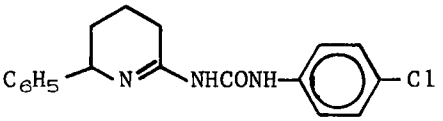
HELMINTH DISEASES

Filariasis - Recent books are concerned with control measures[92] and various aspects of Brugian filariasis.[93] A new screening method involves the transplantation of adult Brugia pahangi into the peritoneal cavity of jirds.[94] The frustration extant in discovering new drugs for treatment of onchocerciasis, for which no convenient laboratory screen exists,[95] is exemplified by trials in humans of metronidazole, tinidazole, mebendazole trichlorophone, oxamniquine, and pyrantel pamoate. None of these agents showed evidence of substantial activity.[96] Reports of the effects of diethylcarbamazine,[97,98] suramin,[98] and levamisole,[99] given transepidermally[98] or directly on the eye,[97,99] offer encouragement that more useful methods of administration of known filaricides may be found.

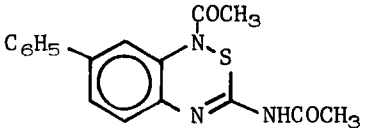
Other Nematodes - Recent reviews cover chemotherapy in both human[100] and veterinary[101] practice. Treatment of 400 patients infected with Dracunculus medinensis (Guinea worm) with thiabendazole was successful.[102] From extensive laboratory comparisons[103] it was concluded that flubendazole and mebendazole possessed comparable antiparasitic properties, but that flubendazole was less toxic for some hosts. Preliminary reports indicate that tioxidazole (13) is broadly active against gastrointestinal nematodes in laboratory animals, sheep and horses.[104] A variety of structures, including 14,[105] 15,[106] and 16,[107] have appeared in the recent patent literature claiming nematocidal activity.



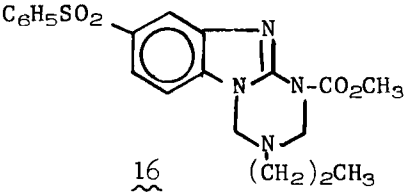
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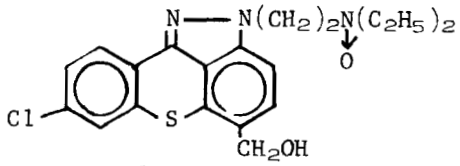


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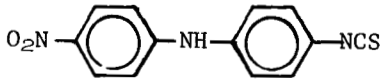


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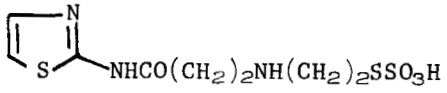
Schistosomiasis - The 7th International Congress of Pharmacology included a symposium on the chemotherapy of schistosomiasis[108] where work with IA-4,N-oxide (17),[109] the diphenylamine 18,[110] oxamniquine, and praziquantel[111] was reviewed. Also addressed were means of reducing the mutagenic liabilities of drugs.[108] No mutagenic activity was found for praziquantel in 2 studies.[112] The 4th volume of the mammoth bibliography[113] was published. A very rapid, new screening method using Schistosoma mansoni in mice was devised[114] and validated with known schistosomicides. Activity against Schistosoma mansoni in mice and monkeys was demonstrated for the thiazole 19.[115] Other structures from the recent literature with antischistosomal activity include the fosfomycin derivative 20[116] and 21.[117]



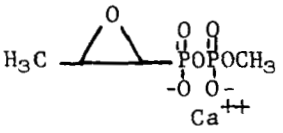
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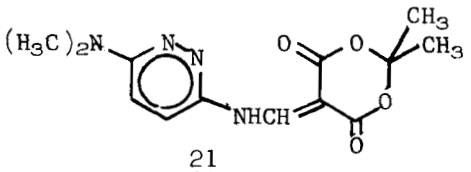
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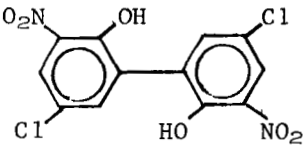
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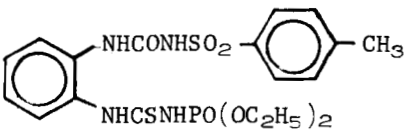
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Other Trematodes - Recent books concerned primarily with fascioliasis include discussions on integrated control strategies,[115] chemotherapy,[119] and collected abstracts of publications on nitroxynil.[120] Cure rates of 73-90% were reported for 95 patients infected with Paragonimus uterobilateralis after a single 2 mg/kg dose of meniclopholan (22).[121]

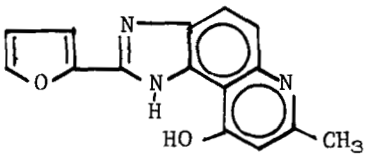
Cestodes - The problems of human diphyllobothriasis[122] and echinococcosis[123] were reviewed. Exceptional anticestode activity was reported for praziquantel,[124] diuredosan (23),[125] furodazole (24),[126] and the niclosamide analog 25.[127]



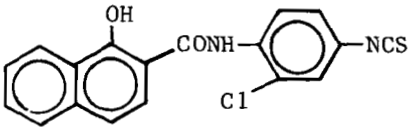
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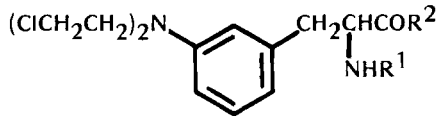
Chapter 14. Antineoplastic Agents

Allen R. Kraska, Pfizer Inc., Groton, Connecticut 06340

Introduction - The annual literature covering chemotherapy and immunotherapy of human neoplasms has more than doubled over the past 5 years. The resulting increased medical awareness concerning the therapeutic or palliative effects of agents administered either singly or in combination has served to increase their use. There is hardly a cancer patient who is not referred to an oncologist practicing several protocols of adjuvant therapy. This increased awareness in the applications and limitations of available antineoplastic agents has also served to stimulate clinical trials with new agents and more basic research into their discovery. Several general reviews and texts of the clinical experiences with chemotherapeutants and immunotherapeutants have recently appeared.[1-10] This is in addition to specific reviews on the anthracycline antibiotic daunomycin,[11] platinum complexes,[12] immunotherapy of cancer with levamisole,[13,14] Corynebacterium parvum,[15] BCG[16] and interferon,[17-21] the biological activities of muramyl dipeptide,[22] and inhibitors of chemical carcinogenisis.[23,24] Also appearing this past year were fundamental reviews of the biology of cancer invasion and metastasis,[25] concepts of cell cycle kinetics in cancer chemotherapy,[26] and new systems for detection of antitumor agents.[27] An especially interesting survey was published on the choice of animal tumors for experimental studies of cancer therapy and a discussion of their validity as models of clinical cancer.[28] Particular emphasis was placed on the relevance of immunotherapy experiments and the type of tumor and species studied. The remainder of this chapter deals with the compounds most studied in the clinic and new entities reported to have antitumor activity in 1978.

Alkylating Agents and Nitrosoureas - Chloroethyl substituted nitrogen compounds and related types of alkylating agents continued to be one of the most widely used class of compounds for the treatment of cancer. Almost all of the new adjuvant chemotherapy protocols have at least one cycle of cyclophosphamide, bis-chloroethyl nitrosourea (BCNU), cyclohexyl chloroethyl nitrosourea (CCNU), methylcyclohexyl chloroethyl nitrosourea (MeCCNU) or the more recently studied 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU), a water soluble nitrosourea. ACNU behaves like the lipid soluble nitrosoureas in vivo[29] and has been found to be of benefit in induction therapy for small cell carcinoma of the lung and metastatic pulmonary tumors.[30] Several clinical trials have been conducted with Peptichemio (PTC; 1), a mixture of six synthetic peptides of m-L-phenylalanine mustard (m-SL). This mixture of alkylating agents has been thought to have an antimetabolic effect because of the peptide group. Treatment with 1-1.5 mg/kg/day for 3-5 days (i.v.) for several cycles has been used for the induction phase of therapy in a variety of solid tumors.[31] With the same treatment regimen in children with advanced neuroblastoma, 11 of 12 patients showed both objective and subjective improvement, with complete remission seen in two of them.[32] Isophosphamide (2), having activity similar to cyclophosphamide, is converted to its active form (3) in vivo by C_4-hydroxylation. In an effort to study preactivated derivatives of 2, analogs were C_4-hydroperoxylated.

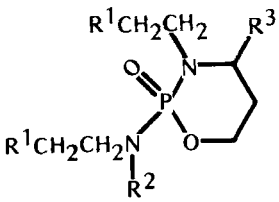
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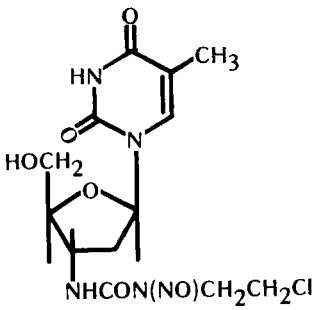
1

R^1	R^2
H–	–(NO_2)Arg·Nval
Ser·p–FPhe–	–OH
Pro–	–p–FPhe
p–FPhe–	–Asn
m–SL·Arg·Lys–	–His
p–FPhe·Gly–	–Nval

These C_4-hydro-peroxides were as effective for activation and were found more stable than the C_4-hydroxyl derivatives.[33] Compound 4 (p.o.) was found to be the most effective against L1210 leukemia. Chloroethyl and methylnitrosourea derivatives of thymidine were prepared and tested for their relative alkylating and carbamoylating activities. Based on the experience with streptozotocin and chlorozoticin, active compounds with reduced bone marrow toxicity possess high alkylating and low carbamoylating activities. Compared with BCNU, 5 had twice the alkylating activity with approximately equal carbamoylating activity, and preliminary *in vivo* work suggested the nucleoside structure enhanced the nitrosourea activity.[34] The antitumor effects of CCNU *in vivo* are thought to be due to hydroxylated metabolites arising via liver enzymes. Selective deuteration of the cyclohexane ring was shown to alter the ratio of 2-, 3- and 4-hydroxylated metabolites; however, the various deuterated analogs demonstrated antitumor activity similar to CCNU against TLX-5 lymphoma in mice.[35] A peptidyl nitrogen mustard Cbz-L-Pro-L-Leu-Gly-L-Pro-Gly-$NHC_6H_4N(CH_2CH_2Cl)_2$ (6) containing a L-Leu-Gly linkage was prepared to exploit tumor-associated collagenase, an enzyme known to cleave this peptide bond. The resulting Gly-L-Pro-Gly-$NHC_6H_4N(CH_2CH_2Cl)_2$ was shown, as expected, to be six times more toxic in mice than 6, but activity against murine sarcoma 180J tumors gave disappointing results. Mouse liver homogenates, however, were also found to produce the desired cleavage, which explained the lack of tumor-specific activity.[36] Quantitative structure activity relationships for a large number of nitrogen mustards were determined. Their rate of hydrolysis was found to correlate with their toxicity and activity against L1210 and P-388 leukemias and Walker 256 tumors. The lipophilic requirements were found to be higher in the case of Walker 256 tumors, suggesting solid tumors may require more lipophilic drugs.[37]

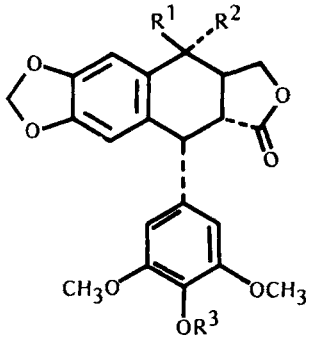


	R^1	R^2	R^3
2	Cl	H	H
3	Cl	H	OH
4	OSO_2CH_3	CH_3	OOH

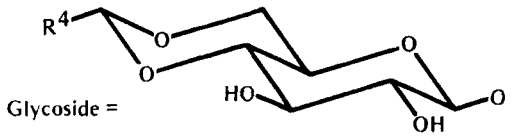


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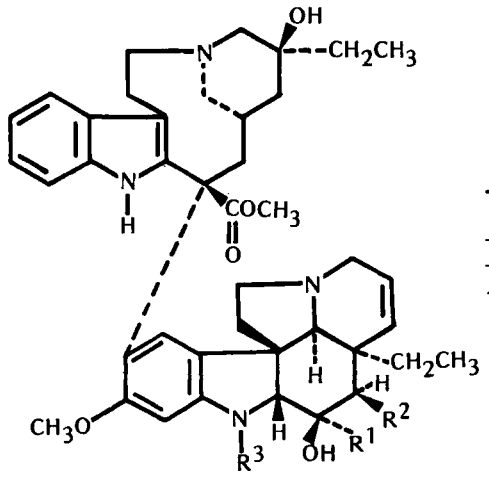
Natural Products and Semi-Synthetics - Several naturally occurring compounds or their derivatives have received considerable attention this past year because of their clinical potential. The epipodophyllotoxins, VP-16,213 (7) and VM-26 (8), known to be potent inhibitors of cell division, were found not to be inhibitors of microtubule assembly as are



	R^1	R^2	R^3	R^4
7	Glycoside	H	H	CH_3
8	Glycoside	H	H	2-thienyl
9	H	OH	CH_3	–
10	OH	H	CH_3	–

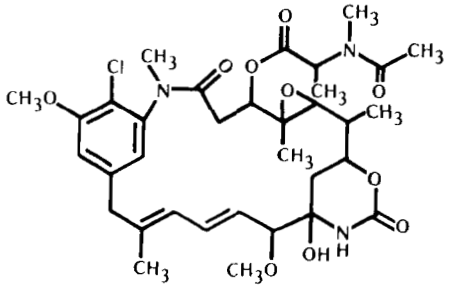


podophyllotoxins (9) and epipodophyllotoxins (10) that are without the glycoside moiety. It was suggested that VP-16,213 and VM-26 are effective during the S or G_2 phase of the cell cycle, whereas the others act in the M phase.[38] A number of clinical trials have been conducted showing that VP-16,213 is one of the most active in the treatment of small cell lung carcinoma.[39-41] Deacetylvinblastine amide (vindesine, VDS; 11) has undergone several successful Phase I/II evaluations, primarily in the treatment of leukemia and lymphoma.[42-44] VDS has been shown to resemble vincristine (VCR; 12) rather than vinblastine (VLB; 13) in animal tumor models, but appears less neurotoxic than VCR.[45] VDS given i.p. inhibited Ridgeway osteogenic sarcoma and Gardner lymphosarcoma, whereas VLB exhibited no activity against these tumors.[46] Maytansine (14),[47] an ansa macrolide 100 times more potent than VCR for blocking mitosis, is also undergoing Phase I/II evaluation.[48] Four new C-3 esters and six new C-9 ether derivatives were prepared to determine the structural requirements at these positions for antileukemic activity.[49] The C-3 ester was found necessary for anti-P-388 leukemia activity, but changes in the structure

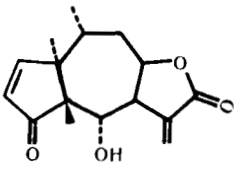


	R^1	R^2	R^3
11	$CONH_2$	OH	CH_3
12	$COOCH_3$	$OCOCH_3$	CH_3
13	$COOCH_3$	$OCOCH_3$	CHO

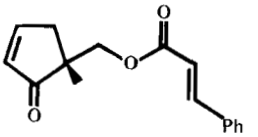
of the ester did not significantly alter activity. Ether derivatives at C-9 were not as effective as the free hydroxyl. In an effort to determine what portion of the tricyclic ring system of helenalin (15) was responsible for its cytotoxicy, the α-methylene lactone was opened with sym-dimethylethylene diamine in a Michael-type addition to form a cyclic lactam. This type of structure was active against a Walker 256 ascites tumor when the free hydroxyls were converted to cinnamate esters.[50] Hydroxymethylcyclopentenones corresponding to the A ring of helenalin were also active against Walker 256 ascites tumor when converted to either a cinnamate (16) or 3,4,5-trimethoxybenzoate ester.[51] Some structural requirements for another class of naturally occurring antineoplastics, the quassinoids, (17) were studied, and it was determined that the epoxymethano bridge and C-1 and C-12 hydroxyls are necessary for biological



14

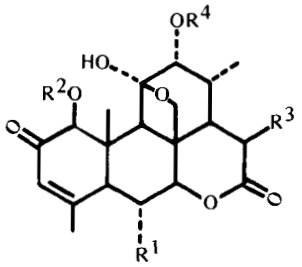


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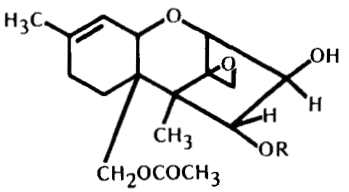


16

activity.[52] The therapeutic index of anguidine (18) was increased when the 4-acetate was hydrolysed with mild base to 15-acetoxyscirpenol (19). A T/C (mean survival of treated x 100/mean survival of control) of >200 was seen in a P-388 lymphatic leukemia assay after 9 daily doses of 0.2-0.4 mg/kg, which was 2-4 fold lower than the maximum tolerated dose.[53]



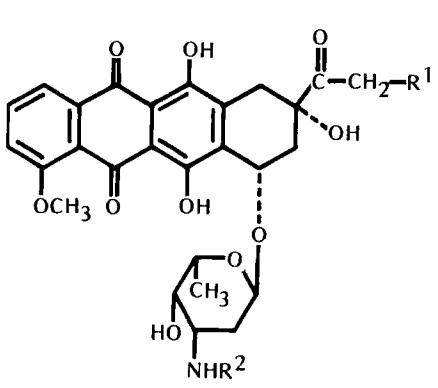
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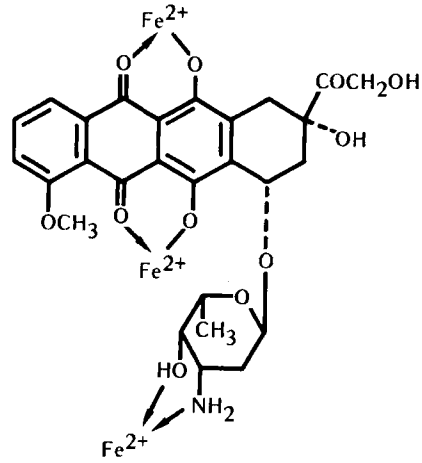
18: R = $-COCH_3$
19: R = H

Anthracyclines - Adriamycin (ADR; 20) has continued to be the dominant force in the expanding use of chemotherapeutic agents. Its wide spectrum of activity coupled with its dose-limiting cardiac toxicity continue to stimulate the search for structurally modified agents with improved biological profiles. Recent reviews on daunomycin (DNR; 21),[11] ADR and related antibiotics[54] have appeared that discuss their chemistry and pharmacology. N-Trifluoroacetyladriamycin-14-valerate (AD-32; 22), unlike the basic anthracyclines, ADR and DNR, has been shown not to

intercalate with DNA and must, therefore, possess a different mechanism for its cytoxicity.[55,56] In comparative studies with ADR, AD-32 at 40 to 60 mg/kg (i.p.) resulted in cures of up to 90% with no early deaths.[57] A similar increased lifespan (ILS) was observed for ADR at 4 mg/kg, but only an occasional survivor past 60 days was observed.[58] Four daily doses of 20-90 mg/kg (i.p.) of AD-32 produced no drug induced deaths in BDF_1 mice after 60 days, whereas a single 20 mg/kg (i.p.) dose of ADR was 100% lethal [mean survival time (MST) of 9 days].[59] The 14-diethoxyacetoxy derivative of daunorubicin (RP 33921; 23) also showed superior antitumor activity versus ADR in L1210 leukemia with a greater than two-fold increase in therapeutic index.[60] 4-Demethoxy-adriamycin and 4-demethoxy-4'-epiadriamycin were synthesized and found to be 10 times more potent than ADR in L1210, P-388 and Gross leukemias.[61] The cardiac toxicity of



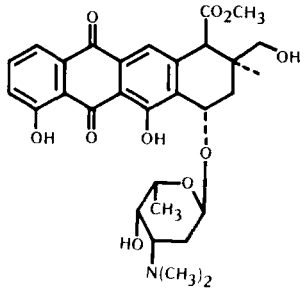
	R^1	R^2
20	OH	H
21	H	H
22	$OCOC_4H_9$	$COCF_3$
23	$OCOCH(OC_2H_5)_2$	H



24

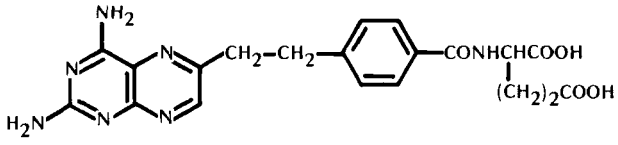
ADR has been reported to be associated with its ability to inhibit both cardiac Na-K dependent adenosine triphosphatase enzyme and cellular ion transport. The presence of calcium reverses these phenomena, and for this reason it has been hypothesized that a chelate of ADR might not be cardiotoxic. Quelamycin (24), a triferric chelate of doxorubicine, was prepared and found to retain antitumor activity and at the same time display less haematologic and cardiac toxicity in experimental systems.[62] Objective responses were observed in bronchogenic carcinoma and ADR-resistant osteogenic carcinoma in a Phase I trial of quelamycin at doses of 125 to 175 mg/m^2; however, in contrast to what was observed in experimental systems, acute iron toxicity and acute cardiotoxicity were also observed.[63] Carminomycin, a 4-hydroxy derivative of DNR reported less cardiotoxic than ADR, was found to be strongly synergistic with cyclophosphamide against L1210 leukemia in mice.[64] An ILS of 63-331% over controls was observed with the combination; neither drug given alone produced an ILS greater than 116. The 13-deoxy analogs of ADR and DNR were prepared and shown to possess similar efficacy and potency to the parent compounds.[65] These analogs, which are also reported to be less cardiotoxic, resemble aklavin (25), whose structure has recently been determined.[66] A quantitative structure activity relationship was obtained for aromatic substituents in the benzylhydrazone moiety of rubi-

dazone, the 13-benzhydrazone derivative of DNR. Cardiotoxicity was found to decrease with increasing lipophilicity.[67]

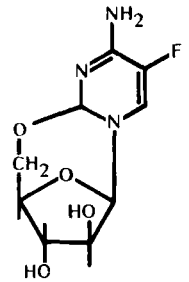


25

Purine, Pyrimidine and Folate Antagonists - High dose methotrexate (MTX) followed by folinic acid rescue has found application in a wide variety of tumors. Thymidine (TdR) has also recently been reported effective as a rescue agent for high dose MTX.[68] As well as being useful in MTX therapy, TdR and other pyrimidine nucleosides were found to enhance 5-fluorouracil (FU) activity against advanced CD8F1 murine breast tumors. The most effective combination produced 70% greater inhibition than FU alone.[69] TdR was also shown to reverse bone marrow toxicity in L1210 leukemia bearing mice treated with FU.[70] The therapeutic index for FU treatment of human cancers has been increased with TdR treatment in several Phase I studies.[71-73] A broader spectrum of antitumor activity was observed versus MTX for a new folate analog, 10-deaza-aminopterin (26). At equal doses, 26 and MTX had increases in lifespan of 171.2% and 149.8% against L1210 leukemia, 64% and 20.9% against Ehrlich ascites carcinoma, and 159.6% and 64% against sarcoma 180.[74] Anhydro-ara-5-fluorocytidine (27), administered in weekly doses of 33-40 mg/kg (i.v.), had significant antitumor activity, 9/17 responses, in patients with adenocarcinoma of the stomach and pancreas.[75]



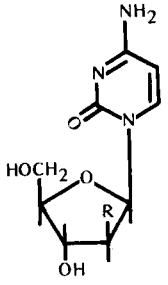
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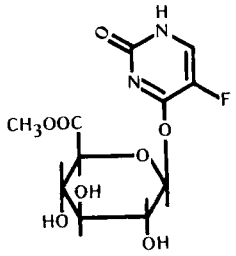
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A convenient synthesis was reported for 1,3-bis(tetrahydro-2-furanyl)-5-fluorouracil (Thf_2-FU), whose toxicity and antitumor activity were compared with Ftorafur (Thf-FU) and FU.[76] The oral LD_{50} in mice was approximately 3-fold greater than that observed for Thf-FU, which was much less toxic than FU. Thf_2-FU slowly hydrolyzed to FU in vivo and showed significant antitumor effects at 0.15-0.45 mmol/kg (p.o.) against Ehrlich and AH-130 carcinomas, sarcoma 180, Yoshida sarcoma and Walker 256 carcinosarcoma. The most active in a series of 1-alkylcarbamoyl derivatives of FU was the 1-t-butylcarbamoyl compound, which gave an ILS_{30} of 8.4 mg/kg.[77] 2'-Azido-and 2'-amino-2'-deoxy-β-D-arabinofuranosylcytosine

(cytarazid; 28 and cytaramin; 29) used at 40 and 75 mg/kg (b.i.d. for 2 days, 24 hr after tumor inoculation) resulted in long-term survival (>120 days) of L1210 leukemia bearing mice. This efficacy was attributed in part to cytarazid and cytaramin not being susceptible to enzymic deamination under conditions where ara-C would be converted to the inactive ara-U. [78] Concomitant dosing of glucose (5000 mg/kg; i.p.) and methyl 1-(5-fluoro-1H-2-oxopyrimidin-4-yl)-β-D-glucopyranuronate (FU-O-G, 30, 400 mg/kg; i.v.) in mice challenged with L1210 leukemia increased the ILS to



28; R = N_3
29; R = NH_2



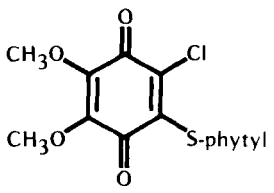
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63% from the 20% observed when FU-O-G was administered alone. The lowering of the pH in tumor tissue by glucose and subsequent increases in the pH-dependent β-glucuronidase activity in that tissue were used to explain the enhancement of FU-O-G.[79]

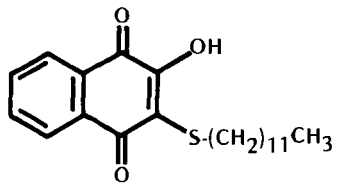
Metal Complexes - The clinical use of the inorganic complex cis-dichlorodiaminneplatinum(II) (DDP) has continued to expand. Its broad spectrum of antitumor properties coupled with its limiting toxic effects have continued to prompt efforts at understanding the mechanism of action and improving the therapeutic index of this type of drug. The synthesis of this class of agents has been reviewed[80] along with the physical properties of their interactions with nucleosides and polynucleotides.[12] Additional evidence has been presented for the nucleophilic displacement of chloride or other carrier ligands by guanine bases in vivo as the mechanism of DDP activity.[81,82] The cis-, trans-d-, and trans-l-1,2-diaminocyclohexane (dach) platinum complexes have been prepared and compared in a variety of tumor systems. It was found that the leaving group as well as the geometry of the dach was important for activity, and Pt (oxalato)(cis-dach) was the most efficacious against murine sarcoma 180 (ED_{90} 0.72 mg/kg) with a therapeutic index range of 14-42.[83] Rhodium and iridium complexes have also been studied for their antitumor effects; bis-1,5-cyclooctadiene (COD)-dichlorodirhodium, acetylacetonate (acac) norbornadiene rhodium and IracacCOD produced cures at approximately one-fourth their toxic doses in mice bearing Ehrlich ascites tumors.[84] Complexes of 6-mercaptopurine and thioguanine with platinum and palladium displayed less activity than the parent purine under the same conditions.[85] Gallium nitrate, a heavy metal compound active in animal tumor systems and shown to be nonmyelosuppressive, has provided responses in initial clinical studies in Hodgkin's disease, lymphoma, ovarian cancer and osteogenic sarcoma.[86,87]

Miscellaneous Synthetic Agents - A series of antimetabolites of coenzyme Q_{10} inhibited human cancer cell lines 4265 and $K562_c$. A % T/C of 923 was

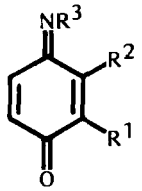
observed with the phytyl analog (31) at 50 mg/kg in Walker carcinosarcoma bearing rats.[88] The antitumor effect was found to correlate with ability to inhibit succinoxidase and NADH oxidase activity. A similar study was done with 2-alkylmercapto-1,4-naphthoquinones, the most active in this



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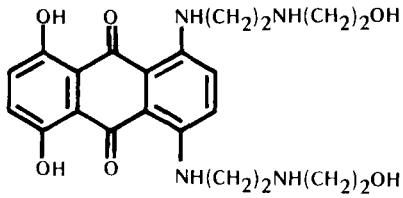


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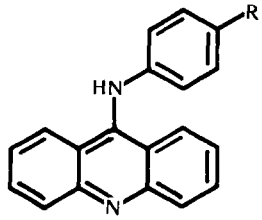


	R^1	R^2	R^3
33	H	Cl	OH
34	Cl	H	4-Me_2NPh-

case being the 2-hydroxy-3-dodecyl analog (32).[89] The most favorable activity and toxicity for nitrogen containing coenzyme Q analogs in mice challenged with sarcoma 180 was observed for the oxime 33 at 20 mg/kg and imine 34 at 90 mg/kg (% T/C of 304 and 256, respectively).[90] Another quinone structure, 1,4-dihydroxy-5,8-bis[[2-(hydroxyethyl)amino]ethyl] amino-9,10-anthracenedione (35) demonstrated potent activity against P-388 leukemia (% T/C of 299 at 0.5 mg/kg with 4/6 cures) and B-16 melanoma (% T/C of 503 at 1 mg/kg with 7/10 cures).[91] The effects on antitumor activity of a variety of substituents in the 9-anilino ring of 9-anilinoacridines have been studied. The substitution must be in the



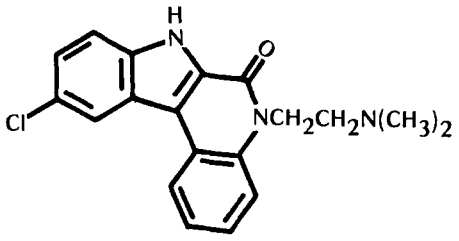
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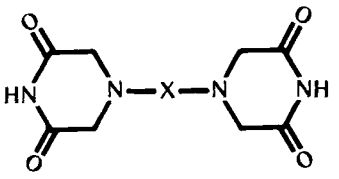
36; R = COOH
37; R = CH_2CH_2COOH

para position, the most potent in L1210 leukemia being carboxyl (36) and carboxyethyl (37), and the activities can be correlated with R_m values obtained from reverse-phase chromatography.[92,93] Further work has been done on the structural requirements for bis-intercalating agents by connecting acridine with other intercalating nuclei.[94] The most active of these bifunctional intercalaters are the polymethylene-linked diacridines, whose in vivo activity in P-388 and L1210 leukemias did not correlate with ability to inhibit those cells in vitro,[95] but was inversely correlated with the rate of agglutination of Con A-treated sarcoma 180 cells. Hydroxyurea with a combination of cyclophosphamide and platinum complexes, such as oxalato (cis-dach) platinum, has been found synergistic in treating L1210 leukemia.[96] The collective cure rate increases from 11% to 53% with inclusion of hydroxyurea in the treatment regimen. A series of amidoximes, structural analogs of hydroxyurea, were evaluated against L1210 and P-388 leukemias, the most active being formamidoxime, acetamidoxime and 2-aminoacetamidoxime.[97] The indoloquinoline 38 was not effective against L1210 leukemia, however it was

active at 25-100 mg/kg (i.p.) or 25-200 mg/kg (p.o.) against Ehrlich carcinoma and sarcoma 180 in mice.[98] Comparison of S-carbamoyl, S-ethylcarbamoyl- and S-chloroethylcarbamoyl-L-cysteine in a variety of mouse and rat tumor models showed the S-ethylcarbamoyl analogs to be the most active (100 mg/kg/day for 6 days, i.p., cured 10/10 mice bearing Ehrlich ascites carcinoma).[99] The cis (39) and trans (40) dioxopiperazines were evaluated with ICRF-159 (41) for their ability to inhi-

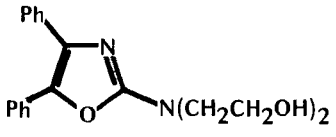


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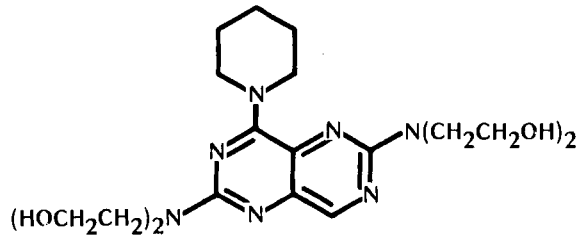


39; X = *cis*-cyclopropyl
40; X = *trans*-cyclopropyl
41; X = $-CH(CH_3)CH_2-$

bit pulmonary metastases of a Syrian hamster lung adenocarcinoma.[100] A structural effect was observed in which the cis derivative was more active than the trans, and 41 actually may have enhanced metastases. There has been a growing literature on prostaglandins (PGs) and their effect on tumor growth and inhibition. Aspirin and indomethacin, inhibitors of PG biosynthesis, have been reported to inhibit growth of a 3-methylcholanthrene(Meth A)-induced fibrosarcoma.[101] Another inhibitor, flurbiprofen, enhanced the activity of chlorambucil on a chemo-resistant line of Walker tumor.[102] In the case of a murine WHT-NC tumor a reduction in growth and ability to synthesize PGs was observed, as well as increased effects of methotrexate and local radiotherapy.[103] Mechanistic evidence suggesting tumors may prevent the effect of a cellular immune response through PG production was provided when PG biosynthesis inhibitors were found to enhance natural and antibody-dependent cell-mediated cytotoxicity against the tumor cell lines T_{24} and HCV_{29}.[104,105] Another possible role played by PGs produced by tumor cells is to affect platelet aggregation, a phenomena associated with enhanced tumor cell metastasis. Two inhibitors of platelet aggregation, ditazol (42)[106] and RA-233 (43)[107] have been shown to decrease metastasis with Lewis lung carcinoma and Ehrlich carcinoma, respectively. Even C. parvum has been shown to cause thrombocytopenia, a fact which has been implicated in its antimetastatic



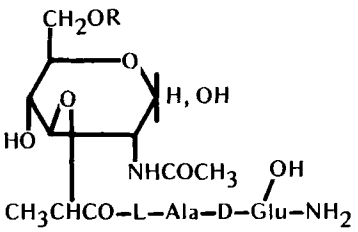
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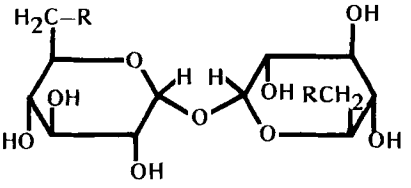
43

activity.[108] Quantitative structure activity (QSAR) relationships have been derived for antitumor activity and toxicity for a large number of aryl-3,3-dialkyltriazenes. The close parallel between the two QSARs suggested improvements in potency would result in increased toxicity.[109,110]

Immunotherapeutics - The theory behind immune modulation and the various experimental models of cancer chemoimmunotherapy have been reviewed.[6,7] Reports have also appeared covering effects on tumor immunotherapy and immunity for the biologicals most often associated with immunomodulation, BCG and C. parvum.[15,16,111] Levamisole, the most widely studied synthetic in this class, was the subject of two reviews.[13,14] Additional reviews in this area were on synthetic immunoregulating molecules in general[112] and the present status of clinical trials in immunotherapy.[8] BCG immunotherapy of L_2C guinea-pig leukemia resulted in increased life expectancy for 66% of treated animals (11/56 survived >15 weeks).[113] A rat mammary adenocarcinoma (13762A) was treated by intratumor administration of C. parvum; local tumor regressed and regional lymph node metastases were destroyed.[114,115] In clinical experiments C. parvum significantly extended survival in stage III lung cancer patients[116] and potentiated the therapeutic effect and reduced the myelosuppression of MeCCNU in stage III malignant melanoma.[117] In efforts to obtain BCG-like effects without using whole cells, work has continued with purified cell walls and cell wall extracts of a variety of microorganisms. Mycobacterium bovis cell wall skeleton (250-500 μg) caused 80% tumor regression in a guinea-pig hepatoma.[118] Extracts of Lentinus edodes (KS-2)[119] and Coriolus versicolor (PS-K)[120] and cell wall preparations of Streptococcus (OK-432)[120] and Norcardia[121] have all been reported to have antitumor activity. The biological activities of synthetic muramyl dipeptide (MDP; 44), the smallest fragment of a cell wall with adjuvant activity, have been thoroughly reviewed.[22] Both lower and higher 6-O-acyl derivatives of MDP have been synthesized.[122] They had adjuvant activities similar to the parent compound, but displayed no antitumor activity. The 6-O-mycoloyl-MDP (45), however, did supress tumor growth in mice bearing a Meth A-induced fibrosarcoma.[123] Cord factor (46), trehelose 6,6'-dimycolate (P3), and lower homologs such as trehelose 6,6'-dicorynomycolate (47),



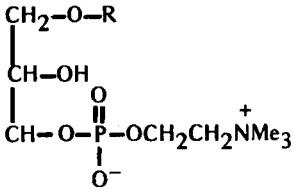
44: R = H
45: R = mycoloyl



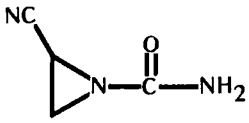
46: R = O-mycoloyl
47: R = O-corynomycoloyl

when combined with a glycolipid and administered intralesionally to strain-2 guinea pigs with line-10 tumors, eliminated tumors and metastases and resulted in specific immunity to tumor re-challenge.[124] Antitumor effects and mechanisms of interferon action have received renewed interest and were subjects of several reviews.[17-21] Ether analogs of lysolecithin (48 and 49) inhibited growth of the mouse ascites tumors,

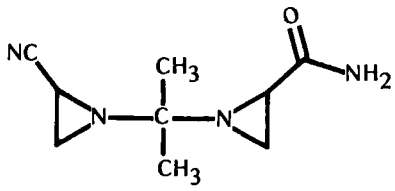
Meth A, Ehrlich and sarcoma 180J, when given as a single i.p. injection from 7 days before to 2 days after tumor challenge.[125] The effect was interpreted as macrophage activation by these agents. 2-Cyanaziridines (50 and 51), a new class of immunomodulating compounds, have been found to increase both the number and mitogenic response of peripheral lymphocytes and increase rejection of a variety of transplanted tumors in rats and mice.[126,127]



48; R = $C_{18}H_{37}$
49; R = $C_{12}H_{25}$



50



51

Drug Delivery - The most widely studied method of reducing toxicity of antitumor agents has continued to be encapsulation in liposomes. The mechanism of drug toxicity reduction and pharmacokinetics of liposome-encapsulated drugs have been reviewed.[128,129] Methotrexate in lipid vesicles enhanced absorption in tumor cells in vitro and resulted in 80% tumor reduction in mice bearing a P1798 lymphosarcoma, a tumor resistant to the same amount of free methotrexate.[130] The unstable nature of aqueous suspensions of CCNU was overcome and the duration of drug action increased, when it was formulated with phosphatidyl choline.[131] Targeting of drugs has also been achieved by linking them to carriers such as DDP and DNA[132] or polylactate,[133] daunomycin and dextran,[134] and mitomycin C and agarose.[135]

Anticarcinogens - A great deal of research has continued toward the goal of inhibiting carcinogenesis. The Laboratory of Chemoprevention of the National Cancer Institute was formed to exploit the unusual activity of vitamin A and its analogs, the most exhaustively studied class of compounds in this area. Reviews have appeared covering general inhibitors of chemical carcinogenesis[23,24] and specific classes such as the retinoids.[136] Ro-11-1430, the trimethylmethoxyphenyl analog of N-ethyl-retinamide, was shown to reduce the radiation-induced transformation of C3H mouse embryo fibroblasts grown in culture.[137] Retinoids have also inhibited transformation caused by sarcoma growth factor produced by murine sarcoma virus-transformed cells.[138] Mechanistically, retinoids have been shown to stimulate deacylation of cellular lipids[139] and to inhibit ornithine decarboxylase.[140] At low doses (25-300 μg/mouse/day), retinoic acid has induced cell-mediated cytotoxicity to allogeneic tumor cells.[141]

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Chapter 15. Immunostimulants

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Introduction - Over the past decade, immunopharmacology has become a viable discipline in its own right. Interest in immunostimulation has been fuelled by advances in bacterial cell wall chemistry, by the rapidly expanding knowledge of endogenous regulators and mediators of lympho-myeloid cell differentiation and cooperation, and by the apparent promise of expermental and clinical tumor immunology. In the context of this brief review, the term "immunostimulant" will be applied in its broadest sense, so as to include immunological adjuvants (substances which are added to antigens in order to increase their immunogenicity), systemic immunopotentiators (pharmacological agents which non-specifically enhance the immunologically specific reactivity of the host), as well as stimulants of non-specific host resistance (pharmacological agents which augment immunologically non-specific effector mechanisms via humoral mediators, lymphocyte subsets, and accessory cells). This rather loose terminology is justified by practical considerations. Clearly, many of the presently available immunostimulants are known to affect multiple target sites and to differ in their activity depending on the mode and schedule of their application.

Bacterial vaccines and crude preparations of bacterial and fungal origin were the subject of intensive investigation and ever-expanding clinical interest. Comprehensive reviews detailing the current status of immunotherapy with BCG[1] and C. parvum[2] have recently appeared. Also, streptococcal culture fluid (OK-432, Picibanil)[3,4] and a protein-bound polysaccharide fraction from Coriolus versicolor (PS-K, Krestin)[5,6] were reported to potentiate immune responses and to inhibit tumor growth by host-mediated mechanisms. Both agents are now available for clinical use in Japan. However, such complex biological preparations are beyond the scope of this chapter which will be restricted to a cursory description of comparatively recent findings with chemically well-defined immunostimulants of exogenous origin.

Peptidoglycans - Several groups continued to characterize the immunopharmacological[7-10] and antitumor[11-12] properties of delipidated cell wall skeleton fragments from various sources. An example is provided by the extensive work on Nocardia water-soluble mitogen (NWSM) which has been reviewed recently.[13] NWSM was reported to enhance T cell-mediated immune reactions if admixed to antigen-oil preparations, to stimulate humoral immune responses if administered together with antigen in oil or saline, to trigger the proliferation of macrophage precursor cells, to exhibit mitogenicity for a distinct subset of B lymphocytes in mice, rabbits and man, to induce circulating interferon in man,[14] and--if attached to oil droplets--to exhibit significant antitumor activity in mice and in patients with malignant pleurisy.[15] It remains to be determined whether the biological profile of NWSM differs from that associated with other peptidoglycans, and if so, whether this can be attributed to distinct structural features of the NWSM. As is the case with other water-soluble peptidoglycans, NWSM exerts its effects on manifestations of cell-mediated immunity only, if

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administered in certain types of lipid vehicles. However, peptidoglycan fragments acylated with lauric acid were found to promote antigen-induced delayed-type hypersensitivity reactions in the absence of oil.[16,17]

Muramyldipeptides - Further progress in the biologic characterization of the synthetic glycopeptide N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP: 1)[18-20] and of many new derivatives has recently been reviewed.[21,22] In several systems, muramyldipeptides are known to substitute for the mycobacterial component of complete Freund's adjuvant (CFA). The structural requirements for the induction of manifestations of cell-mediated immunity were investigated primarily in two different models in guinea pigs: Immunization with MDP-supplemented water-in-mineral oil emulsions containing heterologous protein antigens or azobenzenearsonate-N-acetyl-L-tyrosine (induction of delayed type hypersensitivity),[18-21,23-35] or encephalitogenic proteins and peptides (induction of experimental allergic encephalomyelitis).[36,37] A large series of analogs was compared.[19,20,23,38-41] Derivatives closely related to MDP and/or carrying substituents which may be expected to be readily removed by enzymes (2,[23,35] 3,[29] 4,[27] 5,[20,21,36,37] 6,[20,25,26,29,32,36,37] 7,[27] 8,[23,27] 9,[30] 10,[30] 11,[30] 12,[30] 13,[30,37] 14,[30] 15,[33] 16,[33] 17,[28,33] 18,[21,30] 19,[37] 20,[34] 23[35]), or analogs, where L-Ala is replaced by L-Ser (21)[23,31,37] or L-Abu (22)[35] showed high activity at least in some of the experimental systems investigated. Intermediate or marginal adjuvanticity was found with 24,[18,23,24,37] 25,[29,32,37] 26,[32] 27,[32] 28,[21] 29,[32] 30,[37] 31,[23,27,31,37] and 32.[23,31,32,37] γ-N-methylamidation of D-isoGln or D-Glu-$NHCH_3$ (33,[29] 34[39]), substitution of D-isoGln or D-Glu by D-isoAsn (35),[20,29,37] D-Nle (36),[29] γ-Abu (37),[29] D-Ala (38),[20] or D-Gln (39),[20,21,37] or inversion of their configuration (40,[20,23,27,37] 41[20,27,37]), led to inactive products. Accordingly, 42[20] and 43[29] too were devoid of activity. Also, 44[32] was reported to be inactive. Removal of the essential amino acids of the peptide moiety (45,[19,20,26,36] 46,[20,37] 47,[20,37] 48[20]) or opening or elimination of the intact sugar ring (49,[23] 50,[23,25,37] 51,[25] 52,[20,26] 53,[26] 54[20,26,37]) completely abolished adjuvant activity.

Recently, muramyldipeptides proved to be effective in experimental malaria vaccination of primates which has hitherto depended on the use of CFA. Thus, a significant degree of protective immunity was induced in aotus monkeys[42] and in macaques[43] by Plasmodium falciparum or P. knowlesi merozoites in mineral-oil emulsion supplemented with MDP (1) or Nor-MDP (2), respectively. Full protection against P. falciparum in aotus monkeys was achieved by immunization with mature segmenters mixed with 6-O-stearoyl-MDP (13) and liposomes.[44]

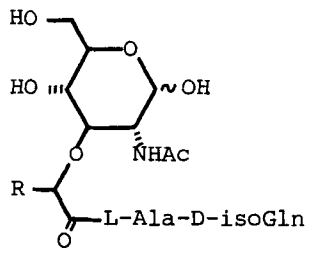
Indeed, lecithin-cholesterol liposomes successfully substituted for mineral-oil emulsion in the induction of delayed hypersensitivity: In guinea pigs, administration of liposome-suspensions containing MDP and ovalbumin induced delayed skin reactivity similar to that observed following immunization in MDP-supplemented incomplete Freund's adjuvant (IFA) or in CFA.[30,45] However, 6-O-acyl-MDP derivatives of appropriate fatty acid chain length (C_{12}-C_{22}, 12, 13, 14), but not C_2-C_8 (9, 10, 11), showed increased effectiveness in this vehicle.[30] Obviously, lipophilic MDP derivatives will to a considerable degree be adsorbed to liposomes.

A:

1 MurNAc-L-Ala-D-isoGln
3 MurNAc-L-Ala-D-isoGln(NH_2)
4 MurNAc-L-Ala-D-isoGln(OCH_3)
5 MurNAc-L-Ala-D-isoGln(L-LysOH)
6 MurNAc-L-Ala-D-isoGln(L-Lys-D-AlaOH)
7 MurNAc-L-Ala-D-Glu-$NHCH_3$
8 MurNAc-L-Ala-D-Glu(OCH_3)-OCH_3
9 6-O-Acetyl-MurNAc-L-Ala-D-isoGln
10 6-O-Butyroyl-MurNAc-L-Ala-D-isoGln
11 6-O-Octanoyl-MurNAc-L-Ala-D-isoGln
12 6-O-Lauroyl-MurNAc-L-Ala-D-isoGln
13 6-O-Stearoyl-MurNAc-L-Ala-D-isoGln
14 6-O-Docosanoyl-MurNAc-L-Ala-D-isoGln
15 6-O-Corynomycoloyl-MurNAc-L-Ala-D-isoGln
16 6-O-Nocardomycoloyl-MurNAc-L-Ala-D-isoGln
17 6-O-Mycoloyl-MurNAc-L-Ala-D-isoGln
18 4,6-di-O-Acetyl-MurNAc-L-Ala-D-isoGln
19 β-Methyl-glycoside of MurNAc-L-Ala-D-isoGln
20 β-p-Aminophenyl-glycoside of MurNAc-L-Ala-D-isoGln
21 MurNAc-L-Ser-D-isoGln
2 nMurNAc-L-Ala-D-isoGln
22 nMurNAc-L-Abu-D-isoGln
23 nMurNAc-L-Ala-D-Glu-$GlyNH_2$

B:

24 MurNAc-L-Ala-D-GluOH
25 MurNAc-L-Ala-D-Glu-GlyOH
26 MurNAc-L-Ala-D-Glu-$GlyNH_2$
27 MurNAc-L-Ala-D-Glu(L-Lys-D-Ala)GlyOH
28 MurNAc-L-Ala-D-Glu-OCH_3
29 MurNAc-L-Ala-D-Glu-D-$AlaNH_2$
30 α-Methyl-glycoside of MurNAc-L-Ala-D-isoGln
31 MurNAc-D-Ala-D-isoGln
32 MurNAc-Gly-D-isoGln



R=-CH_3 : MurNAc 1

R=-H : nMurNAc 2

C:

33 MurNAc-L-Ala-D-isoGln($NHCH_3$)
34 MurNAc-L-Ala-D-Glu($NHCH_3$)$NHCH_3$
35 MurNAc-L-Ala-D-isoAsn
36 MurNAc-L-Ala-D-Nle
37 MurNAc-L-Ala-γ-Abu
38 MurNAc-L-Ala-D-Ala
39 MurNAc-L-Ala-D-Gln
40 MurNAc-L-Ala-L-isoGln
41 MurNAc-L-Ala-L-GluOH
42 MurNAc-L-Ala-L-Gln
43 MurNAc-D-Ala-L-isoGln
44 MurNAc-Gly-D-Glu-GlyOH
45 MurNAc-L-AlaOH
46 MurNAc-D-AlaOH
47 MurNAc-D-isoGln
48 MurNAc
49 Muraminitol-L-Ala-D-isoGln
50 D-Lactyl-L-Ala-D-isoGln
51 D-Lactyl-L-Ala-D-isoGln(L-Lys-D-AlaOH)
52 L-Ala-D-isoGln
53 L-Ala-D-isoGln(L-Lys)
54 L-Ala-D-isoGln(L-Lys-D-AlaOH)

MDP-derivatives with high (A), intermediate/marginal (B) or absent (C) adjuvant activity (as assessed by induction of manifestations of cell-mediated immunity by MDP-supplemented antigen-water-in-oil emulsions in guinea pigs).

6-O-acylation with even longer fatty acids further changed the biologic properties of the parent molecule. Unlike MDP (1) and 6-O-stearoyl-MDP (13),[25] 6-O-corynomycoloyl- (15), 6-O-nocardomycoloyl- (16), and 6-O-mycoloyl-MDP (17) were effective adjuvants for the induction of cell-mediated immunity to allogeneic cells, even if administered together with the antigen in saline.[33] However, 17 was much less effective with respect to induction of humoral antibody formation. On the other hand, 17 showed tumor-suppressive properties and exhibited a greatly decreased pyrogenicity.[33]

Direct coupling of adjuvant active MDP-moieties to poorly immunogenic antigens may provide an opportunity to obviate the use of adjuvant-antigen mixtures in complex vehicles and may further reduce general toxicity by focussing the immunoadjuvant to the target cell clones. In a model experiment, immunogenicity of a MS-2 coliphage coat protein fragment (attached to multichain poly-DL-alanyl-poly-L-lysine) was greatly increased by covalent binding of a natural disaccharide-tetrapeptide from Bacillus megatherium to the antigenic carrier.[46]

The kinetics of cellular and humoral immune responses in guinea pigs immunized with protein antigen in MDP-supplemented IFA differed from those observed with CFA: Antibody responses were faster and higher, but lasted shorter with MDP than with whole mycobacteria.[47] In this model, MDP or active analogs favored the production of specific IgG_2 antibodies.[48] MDP without antigen, in contrast to CFA (or to lipopolysaccharide), did not alter immunoglobulin levels in mice.[48] If given to mice with antigen, however, MDP in saline or in IFA led to an increase in IgG_1 antibodies[48,49] and was claimed to enhance the risk of anaphylactic reactions to the immunizing antigen.[49]

In contrast to the promotion of cell-mediated immunity (at least by water-soluble MDP derivatives), MDP-mediated potentiation of humoral antibody responses does not depend on the use of a lipid vehicle. Thus, MDP in aqueous medium was found to stimulate antibody formation of mice, rats, and hamsters to a variety of antigens, including bovine serum albumin,[24,27,35,50] ovalbumin,[50,51,52] bacterial α-amylase,[25] TNP-ovalbumin,[53] TNP-keyhole limpet hemocyanin,[53] influenza virus subunit vaccine,[54] and, though to a lesser degree, sheep red blood cells[35,49,55] and to the T-independent antigen DNP-Ficoll.[25] The structural requirements for stimulation of antibody formation by MDP derivatives in saline are similar to, though not identical with those found for induction of delayed hypersensitivity by MDP in water-in-mineral-oil emulsions. Thus, 3[29], 4[27], 5[21], 6[29], 7[27], 8[27], 18[21], 21[27], 28[21], and 32[27] displayed both types of activity, while 25[29], 31[27], 33[29], 35[29], 36[29], 37[29], 40[27], 41[27], 43[27] and 45[21] were inactive in either system. On the other hand, 20[34] and 24[24] promoted antibody formation in mice, if administered in saline, but were barely active in the guinea-pig model of delayed-type hypersensitivity. The reverse was true for compound 39.[21]

Increased antibody formation in the presence of MDP was associated with improved carrier priming of T-helper lymphocytes.[53,55] This does not imply, however, that such cells represent the primary target of the MDP effect.

The timing of the MDP application relative to antigenic stimulation appeared to be critical, with maximum adjuvant effects obtained by simultaneous administration of antigen and MDP.[21,35] Moreover, large single doses

of MDP (10 mg/kg) were reported to cause tachyphylaxis in mice, i.e., temporary unresponsiveness to the immunostimulatory effect of a second dose of the same compound, lasting for a period of more than 4 days.[35]

Upon systemic administration, muramyldipeptides were shown to stimulate also non-specific host resistance.[21] MDP and various derivatives (administered in saline) (1, 5, 8, 24, 28[56]; 18, 39[21]) were found to protect adult mice against challenge with K. pneumoniae, while 3, 4, 6, 7, 21, 25, 31, 32, 33, 34, 35, 36, 37, 41, 42, 43[56] and 20[34] appeared to be inactive in this respect. Interestingly, adjuvant activity and protective potential of the compounds were not directly related. Thus, 3, 4, 6, 7, and 21 displayed good adjuvanticity, both in oil emulsion in guinea pigs and in saline in mice, but failed to protect; 24 and 39 exhibited antiinfectious properties, but virtually no adjuvanticity in oil emulsion, although both compounds were found to potentiate antibody formation, when given to mice together with antigen in saline. Protective activity (though not adjuvanticity in oil) was lost by substitution of MDP with a β-p-aminophenyl-aglycone group, but reappeared following cross-linking of the glycosidated MDP with glutaraldehyde.[34] Remarkably, immunologically compromised hosts, i.e. adult thymectomized, irradiated and bone-marrow reconstituted mice could readily be protected against Klebsiella infection by both 1 and 24.[21] Moreover, MDP and some derivatives (1, 5, 24, but not the stereoisomer 31) were found to protect also neonatal and very young mice against bacterial challenge.[57] Compound 5 proved to most effective and was shown to be active even when given by the oral route. Protective MDP derivatives enhanced carbon clearance in adult mice[50,57] and increased the elimination of bacteria from blood in both adult and newborn hosts.[57]

In vitro, MDP was reported to inhibit the migration of guinea pig peritoneal exudate cells without exerting cytotoxicity.[58,59] An adjuvant-inactive stereoisomer had no inhibitory properties.[58] On the other hand, MDP stimulated human monocytes to release lymphocyte-activating factor.[60] Similarly, when purified mouse macrophages were exposed to MDP, they were found to produce a supernatant factor which restored macrophage-deficient lymph-node cell cultures.[35] Moreover, in the presence of MDP, cultured murine spleen cells were reported to release a soluble mediator which stimulated antibody responses of other cultured spleen cells.[61] Production of this factor could be blocked by an antiserum specific for mouse macrophages.[61] Finally, MDP was also capable of inducing the formation of colony-stimulating factor in cultures of mouse macrophages.[62]

In fact, macrophage activation may be responsible for a host of MDP-induced phenomena which have been observed in mouse lymphocyte cultures: MDP and some of its saline-active analogs (1, 4, 5, 8, 24, 28, 39, but not 20, 31, 33, 35, 36) enhanced cell viability and increased the number of background hemolytic plaque-forming cells in mouse spleen cell cultures.[63,64] Also, MDP and nMDP (2) exerted powerful potentiating effects in mixed mouse lymphocyte cultures set up with suboptimal numbers of allogeneic stimulator cells.[35] Immunologic reactivity of T-cell deficient mouse spleen cells from C57Bl/6 or Balb/c athymic donors could be restored by addition of MDP or of its seryl-derivative (21), but not by 24 or 40.[35,65] The relationship of the T-cell substituting and the B-cell mitogenic effects of MDP were not clarified. MDP was repeatedly shown, however, to display distinct mitogenicity for mouse B-lymphocytes from susceptible strains (DBA/2,

Balb/c, AKR, CBA, but not C57Bl/6 or C3H/He).[66] There appeared to be a rather impressive correlation between in vitro activation by different compounds and their in vivo adjuvant activity in saline (stimulation by 1, 4, 5, 6, 7, 8, 21, 24, 28, no stimulation by 20, 31, 34, 35, 40, 43).[66]

Mediator release from accessory cells may also be responsible for side effects of MDP. Pyrogenicity of MDP and of several MDP derivatives was demonstrated in rabbits.[67] Following incubation with MDP, rabbit leukocytes were shown to release pyrogenic material into the supernatant.[21] Moreover, MDP was found to induce a transitory leukopenia[67] reminiscent of the leukocyte disappearance reaction associated with the administration of lipopolysaccharides.[68] On the other hand, MDP is not immunogenic, and the evidence available to date appears to indicate that MDP does not react with naturally occurring or experimentally induced anti-peptidoglycan antibodies from man and rabbit, respectively.[69] In water-in-mineral oil emulsions, though not in saline, MDP was found to induce polymorphonuclear infiltration followed by formation of epitheloid granulomas both at the injection site and in the draining nodes, indistinguishable from the tissue reaction to CFA.[35,70]

In saline, MDP was reported to be absorbed rapidly from the injection site. Following intravenous injection, more than 90% of C^{14}-labelled material was recovered unchanged in urine.[21]

Cord Factors - The interest in immunostimulant properties and antitumor activities of cord factors (6,6'-diesters of α,α trehalose with mycolic acids, corynomycolic acids and nocardomycolic acids) has continued to grow. Comprehensive reviews on the chemistry and the biological activities of cord factors and of synthetic analogs were published.[71-73] Parant et al. reported that a lower homolog of mycobacterial cord factor isolated from Corynebacterium diphtheriae (with corynomycolic acids with chain lengths of C_{28} to C_{36}), as well as some synthetic isomers increased non-specific resistance of mice against infections with Klebsiella pneumoniae or Listeria monocytogenes with the same efficacy as mycobacterial cord factor (with mycolic acid; chain lengths from C_{85} to C_{90}).[74] Synthetic trehalose diesters produced a weaker and more transient inflammatory response in mice, than mycobacterial cord factor. Ribi et al. found that mycobacterial cord factor and some natural and synthetic homologs cause regression of established transplants of syngeneic guinea-pig hepatocarcinoma when combined with endotoxin of Salmonella Re mutant.[75] Yarkoni et al observed regression of established syngeneic murine fibrosarcoma after intralesional administration of 6,6'-di-O-2-tetradecyl-3-hydroxyoctadecanoyl-α,α trehalose, a synthetic cord factor analog.[76]

β-(1→3)-Glucans - A number of naturally occurring β-(1→3)-glucans from various sources (higher plants, fungi, lichen, bacteria and yeasts) are known to stimulate both non-specific host resistance and specific immunologic reactivity and thereby to exert inhibitory effects against transplantable tumors.[77]

Investigation of a partially purified particulate glucan preparation from Saccharomyces cerevisiae cell walls has expanded. Glucan is held responsible for most of the reticuloendothelial-stimulating properties of zymosan.[78] The preparation was reported to activate macrophages as assessed by

size, spreading, adherence, chemotactic mobility and morphologic surface characteristics[79] and to induce a marked but spontaneously reversible granulomatous response in the liver of rats.[80] Glucan-induced enhancement of macrophage and granulocyte production was reportedly mediated by release of colony-stimulating factor from macrophages.[81] In addition, i.v. administration of glucan led to a treatly increased serum lysozyme concentration and enhanced clearance of colloidal carbon in rats.[82] In glucan pretreated mice survival upon challenge with Staphylococcus aureus was enhanced.[82] Similarly, glucan also promoted survival of leukemic mice with experimentally induced staphylococcal infections,[83] and afforded non-specific protection against Sporotrichum schenckii,[84] C. albicans,[85] Cryptococcus neoformans and Mycobacterium leprae.[86]

Dependent on the timing, pretreatment of donor mice with glucan either enhanced or suppressed the response of cultured mouse spleen cells to Concanavalin A and to lipopolysaccharide.[87] Glucan-activated peritoneal macrophages from pretreated donors showed increased cytostatic activity for anaplastic mammary carcinoma[87] and RI leukemia cells.[88] Accordingly, growth of several tumors (s.c. transplanted adenocarcinoma BW10232, anaplastic mammary carcinoma 15091A, melanoma B16 and AKR lymphocytic leukemia,[89] as well as i.v. injected methylcholanthrene-induced fibrosarcoma[88]), was inhibited in glucan-treated mice.[89] Interestingly, proliferation of melanoma B16 was suppressed by glucan treatment both of normal and athymic mice,[87] indicating that a T-lymphocyte independent, presumably macrophage-mediated mechanism appeared to be operative in these hosts. Concurrent glucan and cyclophosphamide therapy had a significant synergistic therapeutic effect in experimental acute myelogenous Shay leukemia of Long Evans rats and in syngeneic lymphocytic leukemia of AKR mice, and led to a considerable reduction in hepatic metastases.[90] Apparently, neither whole body irradiation nor cyclophosphamide treatment markedly interfered with the ability of glucan to render macrophages cytotoxic.[91] Glucan was also shown to exhibit considerable immunoadjuvanticity. Thus, concomitant administration of glucan and poorly immunogenic irradiated L1210 cells in syngeneic mice,[92] or of glucan and glutaraldehyde-treated Shay cells in rats,[93] conferred resistance to subsequent tumor cell challenge. However, failure of glucan to act as an immune stimulator in antileukemic vaccination[94] or therapy of syngeneic tumor transplants[95] was also reported.

Another group of immunostimulating glucans with anti-tumor activity is derived from mushrooms: Lentinan, isolated from Lentinus edodes (Berk.) Sing, believed to be a β-(1→3)-linked linear glucan, pachyman, from Poria cocos Wolf (Bukuryo), consisting of β-(1→3)-linked glucan chains with β-(1→6)-linked branches, pachymaran, derived from pachyman by Smith degradation, removing the branches and thus converting pachyman to a linear glucan, carboxymethyl- and hydroxyethylpachymaran and -pachyman derived from pachymaran and pachyman by carboxymethylation and hydroxyethylation, respectively.[96]

Treatment of mice with lentinan, pachymaran, carboxymethylpachymaran and hydroxyethylpachymaran B, but not with pachyman and hydroxyethylpachyman A, caused host-mediated regression of transplanted sarcoma 180.[96,97] Also, lentinan was reported to inhibit the growth of transplanted methylcholanthrene-induced fibrosarcoma.[88] However, the substances were ineffective in ALS-treated or neonatally thymectomized mice,[97,98] apparently in marked difference to what has been reported of yeast-glucan.[87] Again in con-

trast to yeast polyglucose, lentinan failed to elicit macrophage cytotoxicity,[99] although others found it active in this respect.[88] Moreover, lentinan stimulated T-helper cell activity[100] and restored suppressed T-lymphocyte help in tumor-bearing mice.[101] Lentinan, pachyman, pachymaran, carboxymethylpachymaran and hydroxyethylpachyman A all strongly augmented the formation of alloreactive murine cytotoxic T-lymphocytes *in vivo*.[102] *In vitro*, lentinan, pachyman, pachymaran and carboxymethylpachymaran were found to enhance the generation of such effector cells.[103] Clearly, there was no correlation with the antitumor effects *in vivo*, where pachyman and hydroxyethylpachyman had been reported to be inactive.[96,97] Also, the demonstrated capacity of activating the alternative pathway of complement appeared not to be correlated with the tumor-inhibitory properties of these compounds.[104] It remains unclear, whether the seemingly different modes of action of yeast and fungal glucans reflect differences in supramolecular structure or the presence of hitherto undetected structural elements.

Recently, attention was drawn to a readily accessible bacterial polyglucose: A well-defined β-(1→3)-glucan with $(\overline{DP}_n)$ 540 from cultured *Alcaligenes faecalis* var. *myxogenes* displayed significant host-mediated inhibitory effects against various transplantable tumors. Hydrolytic degradation products ($\overline{DP}_n \geq 16$) retained some activity.[105]

Synthetic Polynucleotides - Synthetic double-stranded polyribonucleotide complexes (formed by polymerization of mononucleotide-diphosphates by *Streptomyces lysodeikticus* polynucleotide phosphorylase and subsequent mixing of complementary single-stranded polynucleotide chains) still hold important promises for future clinical use. Current advances in the biological characterization of poly A:U and poly I:C have been the subject of comprehensive reviews.[106,107] Both polynucleotide complexes exert marked stimulatory effects on cell-mediated and humoral immunity, and on non-specific host resistance to infections and to tumors. However, poly I:C is more toxic,[108] and also more effective than poly A:U in inducing interferon release *in vitro* and *in vivo*[109] except in man, where little or no circulating interferon is elicited by either one of the compounds.

Recent evidence concurs to implicate the T cell as the main target of poly A:U. The substance was reported to associate with membranes of cortisone sensitive (cortical) mouse thymocytes[110] and to induce the secretion of a helper factor from T lymphocytes.[111] Deficient immune responses in newborn and in aged mice could be greatly improved by poly A:U or by supernatant from poly A:U treated thymocytes.[112] Similarly, as demonstrated in cell transfer experiments, induction of tolerance to a T-dependent antigen could be prevented by poly A:U treatment and abrogated by poly A:U induced thymocyte supernatant fluid.[113,114] Depending on the relative time of poly A:U application, generation of helper or of suppressor cells was found to be favored. When poly A:U was given together with the immunizing antigen, enhanced antibody responses were observed, while suppressor cells were activated if poly A:U was injected prior to antigenic stimulation.[115] Moreover, in the absence of antigen, poly A:U was reported to promote non-specific polyclonal activation of cytotoxic T-lymphocytes,[116] while in the presence of allogeneic cells, alloantigen-specific T-cell mediated cytotoxicity was greatly augmented by poly A:U. Amplification of mixed-lymphocyte culture reactions by poly A:U depended on the presence of adherent cells[117] and was

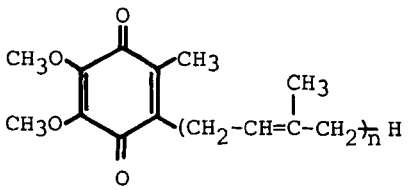
further enhanced by 2-mercaptoethanol.[118] In addition, there is suggestive evidence to indicate that differentiation of both T- and B-lymphocyte precursors may be triggered by poly A:U *in vitro*.[119] Nevertheless, *in vivo*, poly A:U appeared to increase the proportion of T-cell precursors.[120]

Cell-activation by poly A:U may not be restricted to T lymphocytes. Indeed, earlier observations had demonstrated that both poly A:U and poly I:C were mitogenic for B lymphocytes.[121] Very immature B cells were subsequently identified as the susceptible population.[122] It is also documented that poly A:U exerts a profound influence on hemopoietic stem-cell differentiation both *in vivo* and *in vitro*.[123,124] Furthermore, a recent study of poly A:U in experimental murine brucellosis suggested that macrophages are directly or indirectly involved in its antibacterial effects.[125,126]

The cellular targets of poly I:C, on the other hand, may not be identical with those of poly A:U. Thus, poly I:C, but not poly A:U was found to significantly increase natural killer-cell (NK) activity in rat spleen, blood and peritoneal exudate.[127,128] This effect was thought to be mediated by interferon,[129] since interferon has been shown to enhance NK activity by itself[130] while NK activation by poly I:C could be blocked by anti-interferon antibody.[127,128]

The potential clinical usefulness of polynucleotides may have been augmented by measures aimed at prolongation of activity and reduction of toxicity. Increased resistance of poly I:C to hydrolysis *in vivo* was achieved by adsorption to poly-L-lysine and carboxymethylcellulose (poly ICLC).[131] In contrast to poly I:C, poly ICLC proved to be an effective interferon-inducer both in primates[131] and in man.[106] The doses required for this effect were no longer associated with prohibitive side effects.[106] Poly ICLC also displayed increased adjuvanticity, as demonstrated by potentiation of antibody responses to Venezuelan equine encephalomyelitis virus vaccine[132] and to swine influenza virus vaccine[133] in rhesus monkeys, and by enhanced immunogenicity of phase I antigen of *Coxiella burnetii* in guinea pigs.[134] Similarly, inclusion of poly I:C in liposomes potentiated interferon induction and lymphocyte activation, while reducing toxicity.[135] Preliminary clinical trials of poly A:U[136] and poly I:C[137] in cancer patients suggested absence of major side effects. Also, poly I:C appeared to have restored immune reactivity of previously unresponsive patients as assessed by *in vitro* lymphocyte transformation tests. A large controlled clinical trial of poly A:U as an adjunct to surgery in patients with mammary carcinoma is still in progress.[138]

Coenzyme Q - A recent review on biomedical aspects of coenzyme Q (55) has appeared. Earlier work had established that ubiquinones and bacterial phospholipid extracts of various sources enhance phagocytosis,[140] stimulate non-specific host-resistance[141,142] and exert marked adjuvant effects on humoral immune responses to T-dependent and T-independent antigens.[143] Recently, coenzyme Q8 (n=8) was isolated from *E. coli* BM 1135.[144] At 25 mg/kg i.v. 24h before challenge, Q8 protected mice against i.v. challenge with *S. pyogenes*, *E. insidiosa*, *E. coli* 01 and

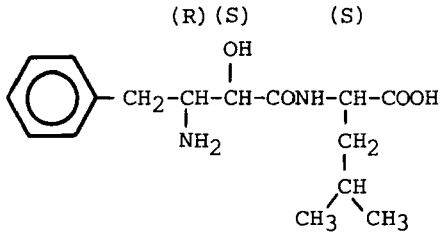


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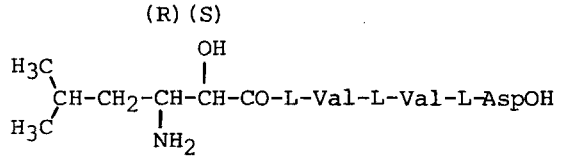
S. typhimurium. Q4, Q6 and Q10 were 5 to 10 times less effective. While Q9 appears to be the preponderant coenzyme Q in mice, in man it is Q10 which was the subject of the earlier research.[140,141] Q8 was found to enhance the phagocytic activity of macrophages and to maintain their activated state.[144,145] Q10 was shown to reverse the deficient primary humoral immune response in old mice.[146] Consideration of properties of antimetabolites of coenzyme Q in certain animal tumor models are beyond the scope of this review.[147,148]

Amphotericin B - Observations on the immunostimulant properties of the clinically used antifungal polyene antibiotic amphotericin B (AmB) and its methylester (AME) were extended. In mice, the drugs augmented antibody formation to TNP-HSA with pronounced enhancement of secondary IgG responses, and promoted contact sensitivity to 2,4-dinitrofluorobenzene.[149,150] The immunostimulant effects of AmB and AME were said to be related to membrane sterol binding. Efficacy appeared to depend on both genetic and dietary factors that regulate cholesterol metabolism.[151] The synergistic action with 1,3-bis(2-chloroethyl)-1-nitrosourea in the therapy of a transplantable AKR leukemia was thought to be due to a host-mediated immunologic effect of AmB.[152]

Protease Inhibitors - Bestatin (56), an aminopeptidase inhibitor isolated from Streptomyces olivoreticuli has been reported to stimulate cellular and humoral immunity against sheep red blood cells in mice[153]. Blastogenesis of peripheral lymphocytes from guinea pigs by concanavalin A and PHA was enhanced.[154,155] Moreover, bestatin appeared to increase the effects of bleomycin and adriamycin in experimental systems.[156] Preliminary clinical studies in tumor patients have been carried out.[157] Another closely related inhibitor, amastatin (57), increased the number of antibody forming cells against sheep erythrocytes as antigen in mice[158].

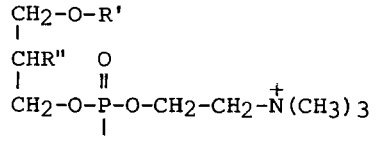


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Lysolecithin Analogs - The immunomodulating effects of a variety of synthetic lysolecithin analogs (58) have been reviewed.[159]



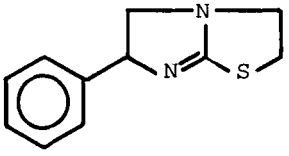
58

58 a : R'=-CH_2-$(CH_2)_{16}$-CH_3, R"=-OH
58 b : R'=-CH_2-$(CH_2)_{14}$-CH_3, R"=-H
58 c : R'=-CH_2-$(CH_2)_{16}$-CH_3, R"=-OCH_3
58 d : R'=-CH_2-$(CH_2)_{10}$-CH_3, R"=-H
58 e : R'=-C(=O)-$(CH_2)_{16}$-CH_3, R"=-H

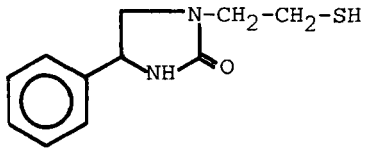
Lysolecithin derivatives were found to increase humoral immune responses in mice to bovine serum albumin and to sheep red blood cells.[159] Compound 58a was most effective. The substance was also shown to suppress the growth of Ehrlich ascites carcinoma, Lewis lung carcinoma and methylcholanthrene-induced fibrosarcoma.[159] Recently, other synthetic lysolecithin analogs were reported to have a marked inhibitory effect on syngeneic transplanted ascites tumors including Meth A, Ehrlich carcinoma and sarcoma 180J. The tumor suppressive effects depended on the substituents R' and R". Compounds 58b and 58c were more effective than derivatives 58d and 58e.[160] The generation of cytotoxic macrophages seems to play a major role in the anti-tumor effects of lysolecithins. Macrophages collected from the peritoneal cavity of mice treated with lysolecithin derivatives killed syngeneic tumor cells with high efficiency.[160] It was suggested that this mechanism might be amplified by a direct cytotoxic effect of alkyl-lysophosphatides, since some of the compounds (58b, 58c) selectively destroyed human leukemic cells in vitro.[161]

Levamisole and Related Compounds - Recent progress in the immunopharmacological and clinical characterization of the immunoregulatory anthelmintic levamisole (59) has been reviewed.[162] Levamisole has been shown to restore compromised responses of polymorphonuclear leucocytes, monocytes, macrophages, and T lymphocytes in a variety of systems both in vitro and, more importantly, in vivo.[163] Thus, monocyte and neutrophil motility and chemotaxis,[164-167] phagocytosis,[162,168,169] lysosomal enzyme release,[162] intracellular killing[162,170] and macrophage proliferation[162,171] could be repaired, enhanced or modulated. T-lymphocyte proliferation,[162,172,173] formation of E-rosettes,[162,174-176] in vitro manifestations of cell-mediated immunity[177,178] and T-dependent antibody plaque formation[179] were reported to be restored. Moreover, in vivo, though not in vitro, levamisole was found to promote differentiation of pre-T cells to mature lymphocytes, thus mimicking the effect of thymic hormones[162]. Levamisole treatment of congenitally athymic mice induced the expression of T-cell membrane markers on spleen cells[179] and inhibited autologous rosette formation.[180] Restoration of genetically defective T-helper and suppressor cell function by levamisole administration in vivo was demonstrated by reappearance of responsiveness to sheep red blood cells in athymic animals,[179] and suggested by suppression of auto-immune disease in NZB and NZB/NZW mice,[181-184] and of Aleutian mink disease.[185] Clinically, cell-mediated immunity was reported to be reconstituted by levamisole therapy in children with primary immune defects.[186]

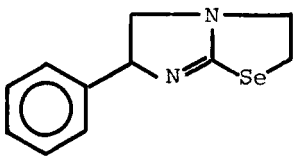
Sera from levamisole-treated mice, rabbits and man were found to yield a dialyzable, complement-unrelated serum factor which did not contain any levamisole metabolites, but which could simulate the biologic effects of levamisole itself.[162] This factor enhanced carbon clearance,[169] stimulated lymphocyte proliferation in vitro,[187] restored immunological reactivity of athymic mice,[179] induced in vitro maturation of mouse pre-T cells,[179] stabilized tumor remission and reduced tumor load in experimental systems.[162] The factor could not be detected in mice which failed to respond to treatment with levamisole; but non-responders reacted to serum factor in a similar way as responders treated with levamisole itself.[162] A perhaps identical serum factor could also be induced by the sulfhydryl compound diethyldithiocarbamate (65),[179] which appears to share some of the immunoenhancing and immunorestorative properties of levamisole if administered in



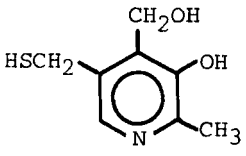
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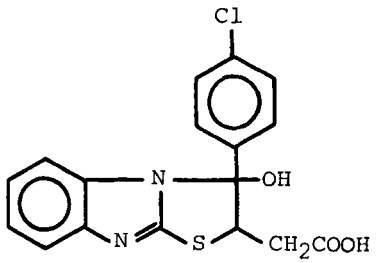
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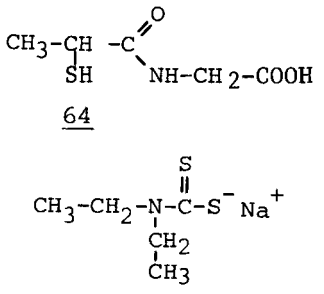
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vivo.[179,188] Interestingly, 2-mercaptoethanol which is known to enhance the viability of cultured lymphocytes[189] to augment their reactivity to antigenic stimulation in the absence of macrophages,[190] and to induce polyclonal lymphocyte activation,[191,192] exerts its activity through a component in fetal bovine serum which is activated by 2-mercaptoethanol and which can be used in place of macrophages or of serum to supplement otherwise macrophage- and serum-dependent lymphocyte cultures.[193] It is tempting to speculate that a similar mechanism is responsible for the activity of levamisole in vivo.

Indeed, levamisole is now thought to be cleaved in vivo to yield dl-2-oxo-3-(2-mercaptoethyl)-5-phenylimidazoline (60),[162] which was reported to be more potent than the parent compound in enhancing carbon clearance in mice[194]. Recent evidence concurs to indicate that both levamisole and thymic factors (Trainin's thymic humoral factor and G. Goldstein's thymopoietin) have similar effects on cAMP and cGMP in lymphomyeloid target cells: increase of cAMP in precursor populations, increase of cGMP in mature effector cells.[162,174,195] In view of the apparent similarities of the in vivo and some of the in vitro effects of levamisole and thymic hormones it was proposed that levamisole might (through the intermediate of a metabolite) activate a serum factor which would in turn combine with a cell membrane receptor for thymic hormones.[162]

The current status of ongoing immunotherapeutic trials with levamisole in immune deficiency disorders, recurrent or chronic infections, chronic inflammatory diseases and cancer has been reviewed comprehensively.[162] Recent trials indicate that levamisole provides significant therapeutic benefits in rheumatoid arthritis.[196-199]

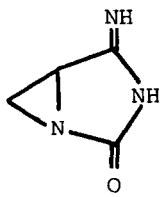
A levamisole analog containing Se instead of S (Se-levamisole, 61) produced a lesser immunopotentiating effect than the parent compound in a murine skin graft model (C3H donors, $B6AF_1$ recipients). However, in a system involving a y-locus difference ($B6AF_1$ male → $B6AF_1$ female), 61 accelerated rejection, while levamisole was found to suppress it.[200]

A related compound, 3-(p-chlorophenyl)-2,3-dihydro-3-hydroxythiazole [3,2-α]-benzimidazole-2-acetic acid (Wy-13876) (63) increased in vitro antibody formation of mouse spleen cells sensitized with sheep red blood cells (SRBC) as measured by a direct plaque assay. The compound did not have a mitogenic effect.[201] It was reported that i.p. administration of 63 (50-150 mg/kg) enhanced the primary and secondary anti-SRBC response after optimal immunization in mice and raised the graft-versus-host-inducing capacity of splenocytes; no influence on the response to type III pneumococcal polysaccharide (a T-cell independent antigen) nor on macrophage cytotoxicity toward SL2 and TLX9 lymphomas could be observed. However, the drug inincreased survival of L1210Ha leukemia-bearing mice, when administered in conjunction with irradiated tumor cells. At 150 mg/kg i.p. one day after tumor transplantation compound 63 exerted an antimetastatic effect in a Lewis lung carcinoma model.[202]

5-Mercaptopyridoxine (62) was reported to enhance the intensity of the secondary reaction in adjuvant arthritic rats and to augment pertussis vaccine edema. Also, it stimulated lymphocyte responses to Concanavalin A.[203] Another sulfhydryl compound, α-mercaptopropionylglycine (64), enhanced antibody formation to SRBC in mice when given for five days before immunization. T- and B-cell mitogen reactivity of lymphoid cells from EL4 leukemia-bearing mice was restored and production of cytotoxic antibody to EL4 increased by the administration of 64.[204]

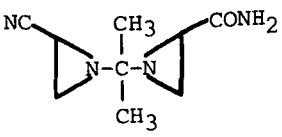
Other Synthetic Immunostimulants

2-Cyanaziridines - 4-Imino-1,3-diazabicyclo[3.1.0]hexan-2-one (66), (BM 06002; Imexon) was reported to inhibit tumor growth in various tumor models in the mouse and rat,[205] and to increase the resistance of mice to chronic infection with Candida albicans; when combined with a subtherapeutic dose of sulfadiazine, compound 66 was found to increase resistance of mice against Staphylococcus aureus infections.[206] Preliminary clinical trials suggested that 66 stimulates delayed cutaneous hypersensitivity reactions and lymphocyte blastogenic response in tumor patients.[207,208]



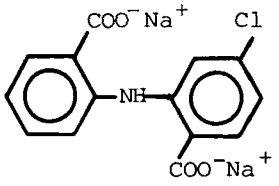
66

2-[2-cyan-aziridinyl-(1)]-2-[2-carbamoylaziridinyl-(1)-propane] (67), (BM 12531; azimexon) also showed therapeutic effects in tumor models and experimental infections in mice.[209,210] Immunostimulant effects of compound 67 were demonstrated by various parameters, including augmented delayed type hypersensitivity reactions in mice, enhanced human T-lymphocyte transformation in vitro, and increased phagocytosis of latex particles by mouse peritoneal cells.[211-213]



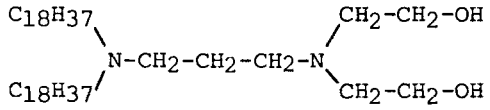
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N-(2-Carboxyphenyl)-4-chloroanthranilic Acid Disodium (CCA) Compound (68) was reported to suppress adjuvant arthritis in rats without anti-inflammatory and immunosuppressive activities.[214] Surprisingly, oral administration of CCA augmented the number of plaque-forming cells (PFC) in the mouse spleen against both a thymus-dependent (sheep red blood cells) and a thymus-independent antigen (bacterial lipopolysaccharide).[215] It was also reported that CCA prevented development of autoimmune kidney disease in NZB/NZW F_1 hybrid mice. CCA treatment inhibited antibody production to double-stranded native DNA and led to decreased amounts of IgG deposited in glomeruli; the Con A responsiveness of thymocytes from CCA-treated donors was found to be increased. It was suggested that prevention of autoimmune kidney disease by CCA might be due to activation of suppressor T-cells.[216]



68

The synthetic alkyldiamine CP 20961 (69), which had been investigated earlier with respect to its interferon-inducing properties,[217,218] was reported to substitute for mycobacteria in CFA in the induction of adjuvant arthritis and the development of cell-mediated and humoral immune responses to heterologous lymphoma cells in rats.[219]



69

Conclusions - Due to the rapidly expanding interest in immunostimulants, there has been a dramatic proliferation of recent papers on immunopharmacology and of molecular entities which have been identified as modulators of the immune response. For obvious reasons, the choice of agents discussed on the foregoing pages had to be arbitrary. Thus, very important classes, such as synthetic polymers with immunostimulating activity, endogenous hormones and mediators, including interferon and small molecular weight synthetic interferon inducers, have not been dealt with in this chapter. A particularly important omission is the intriguing topic of the immunotherapeutic potential of established drugs with a presently defined spectrum of non-immunologic indications. Indeed, inhibitors of prostaglandin synthesis, histamine- and catecholamine-receptor antagonists, as well as some regulators of ion transport may yet reveal their immunoregulatory properties.

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Section IV - Metabolic Diseases and Endocrine Function

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Chapter 16. Chemical Control of Fertility

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Introduction - The results of a survey[1] of married women aged 15-44 for the 1973-1976 period indicated the following major contraceptive choices: "pill" (22%), sterilization (19%, female and male), condom (7%), intrauterine device (IUD) (6%), rhythm (3%), foam (3%), diaphragm (3%). Major trends are the increasing use of sterilization and a somewhat decreasing reliance on the "pill". Approximately 600,000 male and 600,000 female sterilizations are performed annually in the U.S.[2] With compliance, the "pill" is an excellent method of family planning with a failure rate of 1 pregnancy or less per 100 women-years. A beneficial effect to undernourished women is a decrease in menstrual blood loss. Compliance can be a problem and missed medications can result in pregnancy. Although the IUD is not as effective as the "pill", compliance is not a problem if the IUD is properly inserted and remains in place. Associated with the use of the unmedicated IUD is a significant increase in menstrual blood loss.[3] The incorporation of Cu or a progestin in these devices has increased their effectiveness.[4,5] Menstrual blood loss seen with the unmedicated IUD is reduced with Progestasert®, an IUD whose effectiveness approaches that of the "pill".[4] Elective abortion as a method of family planning is increasing as a result of the availability of safe and legal abortion services.[6] There is one elective abortion in the U.S. (1.3 million in 1977) for every three live births.

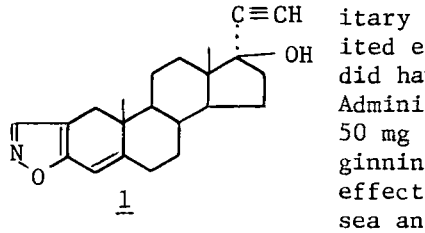
Since the introduction of the "pill" in 1960, the major advances from a medicinal chemical point of view have been the introduction of prostaglandins (PG's) as abortifacients and the medicated IUD's. Neither has yet had the impact of the "pill" but progress has been reported in the development of PG's and IUD's with improved properties. Most of the recent research with sex hormones deals with previously reported compounds. An important finding in the area of luteinizing-hormone-releasing hormone (LH-RH) analogs is that both agonists and antagonists are potential antifertility agents. There are no reports evaluating this class of drugs as human antifertility agents. The immunological approach to fertility control has been reviewed.[7] A review of many approaches is available.[8]

Steroids - Two recent reviews[9,10] summarize the adverse reactions from use of the "pill". The "paper pill", a square of edible cellulose impregnated with the steroids, is advantageous with respect to packaging, transportation, acceptability and storage. The conventional and the "paper pill", each containing 150 μg of levonorgestrel and 30 μg of ethynylestradiol (EE), were equally effective in suppressing ovulation.[11] The EE content of the "pill" was lowered to 30 μg without loss of contraceptive

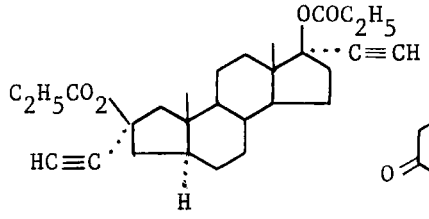
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efficacy.[12] In a modified sequential oral contraceptive dosage schedule, 50 μg of EE administered for 7 days followed by 50 μg of EE and 1 mg of lynestrenol for 15 days resulted in complete inhibition of ovulation.[13] A useful alternative to the pill, especially in women in whom estrogen is contraindicated, is Depo-Provera® (medroxyprogesterone acetate) which when given i.m. every 3 months is an effective contraceptive.[14] This progestin in large continuous doses induced an increased incidence of mammary cancer in beagle dogs, an effect not reported in other animals or women.[15] The drug is not available in the U.S.[16]

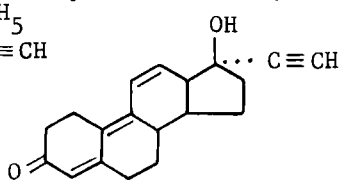
Danazol (1) has been reported to be an oral contraceptive in the rhesus monkey[17,18] and in women.[19] In rats, this substance was a pituitary gonadotropin inhibitor which neither exhibited estrogenic nor progestational activity but did have weak impeded androgen-like activity.[20] Administration of estrogens, either 5 mg of EE or 50 mg of diethylstilbestrol p.o. for 5 days beginning within 3 days of coitus in midcycle, is effective in preventing pregnancy in women. Nausea and vomiting are common side effects. Diethylstilbestrol caused an increased incidence of vaginal adenosis and adenocarcinoma of the vagina and cervix in female offspring of women treated during the first trimester with this drug for other reasons.[21] In China, the estrogen anordrin (2) is taken immediately after coitus for prevention of pregnancy.[22] When EE and dl-norgestrel were administered in combination to 608 women shortly after coitus, one pregnancy occurred



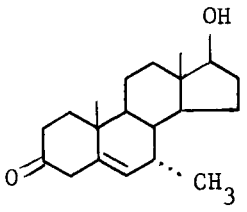
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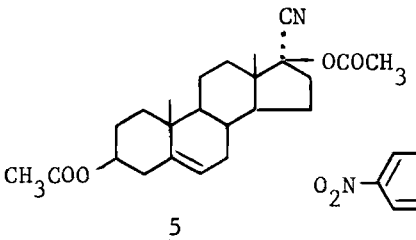
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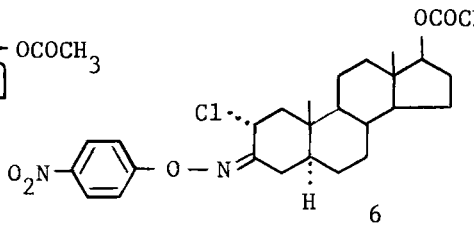
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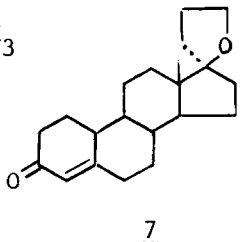
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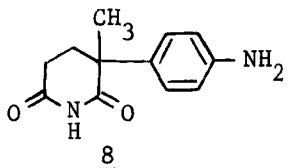


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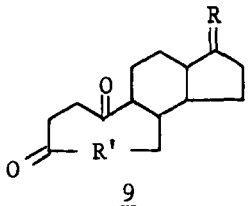
rather than the 12-30 pregnancies expected.[23] The antiprogestin R-2323 (norgestrienone, 3) had low contraceptive efficacy in women.[24,25] It was suggested that the antifertility effects of RMI 12,936 (4) resulted from its antiprogestational activity as well as inhibition of progesterone (P) biosynthesis.[26] The abortifacient activity of the cyanohydrin diacetate 5 in the rat may be the result of its transformation to estrogens in the ovary.[27] ORF 9326 (6) terminated pregnancy in rats when administered p.o. on days 9-12 of gestation.[28] At the effective dose, the compound lacked estrogenic, androgenic, and progestational activities. The spiroether 7

was an estrogen antagonist in mice and ewes but was not estrogenic in rats. When administered to normally cycling rhesus monkeys, it increased the viscosity of the cervical mucus, a possible antiestrogenic effect, and prevented pregnancy.[29]

Steroidogenesis Inhibitors - Aminoglutethimide (8), which blocks the conversion of cholesterol to P, terminated pregnancy in rats[30] but not in baboons.[31] The 5,10-seco steroids 9 irreversibly blocked Δ^5-3-ketoisomerase from Ps. testosteroni in vitro and caused a decrease in the weight of male sex accessory organs of the rat, an effect which could be the result of inhibition of androgen biosynthesis.[32] Azastene (10) was a competitive inhibitor of P biosynthesis in vitro and disrupted pregnancy in both the rat[33] and rhesus monkey.[34] This agent blocked human chorionic gonadotropin-induced prolongation of corpus luteum function and lacked hormonal activity.[35] Trilostane (11), also a competitive inhibitor of 3β-



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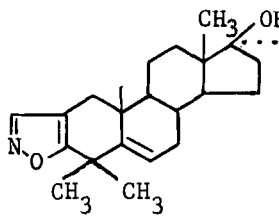
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R=O, $\overset{\diagup\!\!\diagdown}{\underset{H}{\cdots}}COCH_3$

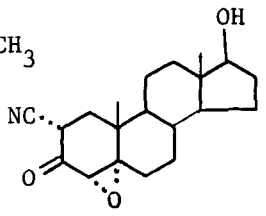
R'=$-CH_2C\equiv C-CH_2$

or $CH=C=CHCH_2$

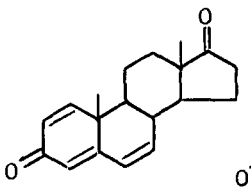
hydroxysteroid dehydrogenase, was more effective in blocking adrenal steroidogenesis than in disrupting pregnancy in the rat[36] and rhesus mon-



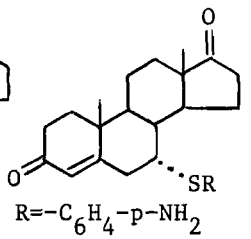
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R=$-C_6H_4$-p-NH_2

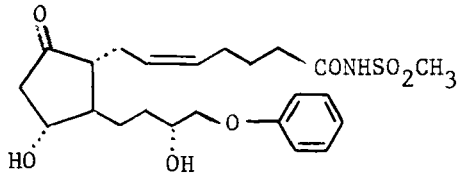
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key.[37] Pregnancy in the monkey was terminated at a time when the ovaries are required for the continuation of pregnancy as well as when the placenta is capable of maintaining pregnancy in the absence of the ovaries. The estrogen synthetase blocker 12 inhibited mating, ovulation, and implantation in rats.[38] Of a series of aromatase inhibitors, 13 was the most active in vitro.[39]

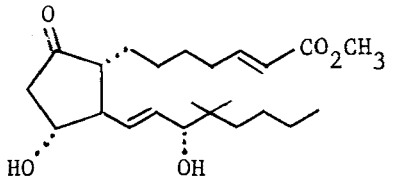
Prostaglandins - $PGF_{2\alpha}$ is available on a limited basis in the U.S. for the termination of second trimester pregnancy by the intraamniotic route. PGE_2 has been approved in the U.S. for the same indication as an intravaginal suppository.[40] The primary action of the PG's in humans is believed to be a direct stimulatory effect on myometrial smooth muscle.[41-45] The decreases in serum P and estradiol levels are considered a consequence and not a cause of the disruption of pregnancy. It should be remembered that pregnancy termination by luteolysis is only possible during early gestation since the corpus luteum is not required for maintenance of pregnancy after 6 or 7 weeks of gestation, the time at which the luteoplacental shift takes place.[46,47] When administered in the mid-luteal phase

$PGF_{2\alpha}$ is not luteolytic in normal cycling women but is luteolytic in rats and other species.[49] The results of an international multicenter study were interpreted to indicate that intraamniotic saline and $PGF_{2\alpha}$ (25 mg repeated in 6 hours) are safe and effective for termination of second trimester pregnancy,[50] and that $PGF_{2\alpha}$ is significantly more effective than saline in the termination of pregnancy within 48 hours. Another interpretation of these results pointed out the hazards of $PGF_{2\alpha}$ and intraamniotic saline and advised that, up to the sixteenth week, dilatation and evacuation is the most advantageous available method.[51] An advantage of dilatation and evacuation is the low incidence of incomplete abortion compared with intraamniotic $PGF_{2\alpha}$ or saline.[51] In second trimester pregnancy a single intraamniotic injection of 2.5 mg of 15(S)-methyl $PGF_{2\alpha}$ methyl ester was slightly more effective than 50 mg of $PGF_{2\alpha}$ in inducing abortion within 48 hours.[52] About one half of the abortions were incomplete with both drugs. In another multicenter study, a 92% abortion rate, 50% of which were incomplete, was achieved within 30 hours after repeated intravaginal administration of 15(S)-methyl $PGF_{2\alpha}$ methyl ester during the second trimester.[53] Vomiting and diarrhea were significantly more common with the PG analog, but the mean incidence of diarrhea and vomiting ranged from 2 to 6 episodes per patient in both studies.[52,53] First and second trimester pregnancy was terminated with 15(S)-methyl $PGF_{2\alpha}$ methyl ester administered as a single intravaginal suppository.[54-56] Intravaginal administration of 16,16-dimethyl PGE_2 was as effective in terminating first trimester pregnancy as vacuum aspiration but was associated with a much higher incidence of side effects. The incidence of side effects was lower than with 15(S)-methyl $PGF_{2\alpha}$ methyl ester.[57] Summaries of the results of clinical evaluation of PG's for pregnancy termination have been published.[58,59]

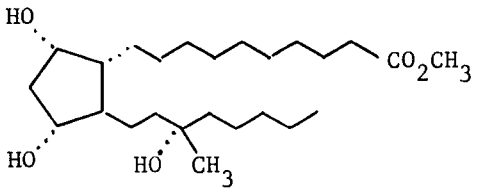
Sulprostone (CP-34,089, ZK 57,671, SHB 286, 14) was 10-30X more active than PGE_2 as an antifertility agent in laboratory studies but equal to or less active than PGE_2 in laboratory tests considered to be predictive of side effects.[60] Administration of this agent i.v., i.m. or by the



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transcervical intrauterine route terminated first and second trimester pregnancies in women.[61-64] A decrease in the incidence of side effects compared with other PG's was reported, a particularly significant result since the i.m. route could be advantageous in the developing countries.[65] A similar favorable separation of activities has been reported in the monkey for ONO-802 (15).[66] This PG terminated early pregnancy in women when given by the transcervical intrauterine route[67] or by pessary.[68] In the latter study, complete abortion occurred in 54 of 63 women; nausea and

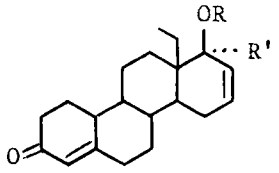
vomiting were reported by one patient. Encouraging results have also been obtained in studies with 16,16-dimethyl PGE_2 p-benzaldehyde semicarbazone ester for termination of first and second trimester pregnancies and with the dihomo PG derivative 16 for termination of abnormal intrauterine pregnancies.[59]

LH-RH Antagonists - Des-Gly^{10}-[Trp^2,Leu^3,D-Ala^6]LH-RH ethyl amide (EA) inhibited ovulation in rats after a single s.c. dose of 1.5 mg/rat on the day of proestrus.[69] [D-Phe^2,Pro^3,D-Trp^6 or D-Phe^6]LH-RH were active at 750 µg.[69,70] Ac-[Pro^1,D-Phe^2,D-Trp^3,D-Trp^6]LH-RH and [D-<Glu^1,D-Phe^2, D-Trp^3,D-Trp^6]LH-RH were active at 200 µg.[71] Aza-amino acid substitution in biologically active peptides has usually resulted in diminished activity. [D-Phe^2,D-Phe^6,Azgly^{10}]LH-RH, however, was 8X as active as the Gly^{10} analog as an inhibitor of LH-RH-induced ovulation in rats; 15 µg/rat completely inhibited ovulation induced by 0.5 µg/rat of LH-RH.[72] The branched pentadecapeptide [D-Phe^2,D-Trp^3,N$^{\epsilon}$ (<Glu-D-Phe-Trp-Ser-Tyr),D-Lys^6]LH-RH, which may be regarded as having two antagonist N termini, was more active as an ovulation inhibitor in the rat when administered s.c. on the day of proestrus than the parent unacylated lysine peptide.[73] On a molar basis, it was 50% more active than the very active [D-Phe^2,D-Trp^3, D-Phe^6]LH-RH. The enhanced activity did not appear to be the result of an increased metabolic stability. Inhibitory analogs may be fitted into the calculated conformation of LH-RH.[74] The antagonist [D-Phe^2,D-Ala^6]LH-RH, earlier shown to be effective as an ovulation inhibitor in normally cycling rats and rabbits, was, at best, minimally effective in Macaca fascicularis.[75] This monkey species may not be a suitable model for evaluation of LH-RH antagonists since it is relatively insensitive to exogenous LH-RH and synthetic agonists. A 90 mg i.m. dose of the inhibitor [D-Phe^2,D-Trp^3,Phe^6]LH-RH induced a statistically significant suppression of LH-RH-induced release of LH and follicle stimulating hormone (FSH) in men.[76] [D-Phe^2,Phe^3,D-Phe^6]LH-RH was inactive at 60 mg in the same test system as was des-His^2,des-Gly^{10}-LH-RH, the latter analog administered i.v. This is the first report of activity of an LH-RH inhibitor in humans. The number of subjects was small, however, and the inhibitor failed to depress basal levels of LH and FSH. To inhibit the reproductive process in the male, depression of basal levels of these hormones would presumably be required.

LH-RH Agonists - LH-RH and its agonist analogs are antifertility rather than profertility drugs in many experimental models despite their inferred profertility properties.[77] LH-RH and three analogs, des-Gly^{10}-[D-Ala^6]-LH-RH EA, des-Gly^{10}-[D-Ala^6,N-Me-Leu^7]LH-RH EA and des-Gly^{10}-[D-Trp^6],N-Me-Leu^7]LH-RH EA, exhibited post-coital contraceptive activity in the female rat when administered during the pre- or post-implantation stages of gestation.[77] The more active agonists in an ovulation induction test were the more active agents in disrupting pregnancy. The observed antifertility effects may be the result of an overstimulation or mistimed stimulation of the hypophyseal-ovarian axis and consequent disruption of the normal series of events required for successful gestation. LH-RH was modestly uterotrophic in the hypophysectomized rat, a theretofore undetected extrapituitary action of the hormone not shared by three agonist analogs.[78] Spermatogenesis was inhibited in male rats administered 100 ng s.c. of des-Gly^{10}-[D-Ala^6]LH-RH EA every 3 days for several weeks.[79] The antifertility activity of agonists is associated with a decrease in gona-

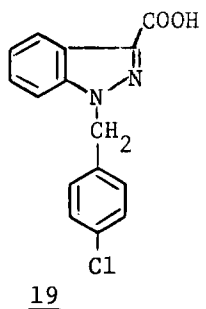
dotropin receptor concentrations in the gonads of both male and female rats.[80-82] Ovulation inhibition in women was achieved by treatment with [D-Ser(t-Bu)^6]LH-RH EA (HOE 766) s.c. daily for 22-30 days at a dose of 5 µg commencing within 3 days of the beginning of menstruation.[83] Premature luteolysis in women resulted from administration of des-Gly^{10}-LH-RH EA s.c. for 5 days during the post-ovulatory phase.[84] Administration of LH-RH at a dose of 250 µg several times on one day between the second and seventh day following ovulation caused premature luteolysis in women.[85] There was evidence of ovulation on laparotomy in 9 of 10 women after pretreatment with EE every 12 hours on days 10 and 11 or days 9-11 followed by one injection of [D-Leu^6]LH-RH EA.[86] If these results are confirmed, a method could be available for improving the effectiveness of the rhythm method. As in the above study,[86] however, it may be necessary to prime the hypothalamic-pituitary-gonadal system by administration of an estrogen prior to the administration of the LH-RH agonist. The observation that cyclo[(β-Ala^1) and (6-Aminohexanoyl^1),D-Ala^6)]LH-RH were more potent agonists than the uncyclized peptides, although only about 1% as potent as LH-RH, supports an "U" shape conformation for LH-RH.[87] Structure-activity relationships of LH-RH and its analogs have been reviewed.[88]

Male Contraception - The most promising approach to male contraception is suppression of spermatogenesis mediated by inhibition of gonadotropin release by the pituitary gland.[89-91] Inhibition of spermatogenesis in men by monthly injections of a combination of Depo-Provera and testosterone enanthate (TE) continues to be studied with the objective of developing the optimum dosage schedule.[92-94] Danazol (1) plus testosterone propionate or TE caused a variable reduction of spermatogenesis in man.[95,96] TE administered i.m. biweekly also did not result in uniform azoospermia in a year-long study.[97] It is not known if azoospermia must be achieved for complete inhibition of fertility or whether oligospermia would be sufficient.[93,94,98] The use of implants containing androgens is not yet practical since the implant would be too large emphasizing the need for more active androgens.[99] The D-homo steroids 17 and 18 are considered possible candidates for use in implants since they are more effective than testosterone in suppressing pituitary gonadotropin secretion and, as a result, have an appropriate balance of peripheral and central activities.[99] Antiandrogens such as cyproterone acetate have not been useful as male antifertility agents since libido is reduced at effective contraceptive doses.[100] The effects of prolonged suppression of spermatogenesis need to be studied and it must be established whether fertility returns upon withdrawal of drug.[97,98] α-Chlorohydrin(3-chloropropane-1,2-diol) is an example of a compound that interferes with sperm maturation.[101] Toxic side effects, particularly in the monkey, precluded trials of the compound in man. The antifertility activity of α-chlorohydrin may be the result of an inhibition of glyceraldehyde-3-phosphate dehydrogenase in spermatozoa. The S(+)-isomer is more active in vitro and in vivo than the R(-)-isomer. In vitro experiments suggest that conversion to the 1-



17 R=COC_2H_5,R'=H

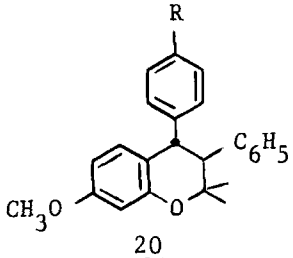
18 R=H,R'=C≡CH



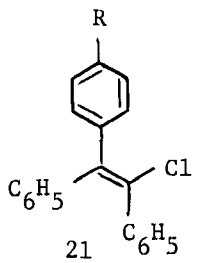
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phosphate is necessary for activity.[102] Some 6-chloro-6-deoxy sugars had a reversible contraceptive action in the male rat and were less toxic than α-chlorohydrin.[103] AF 1312/TS (19) inhibited spermatogenesis in the rat and in the monkey.[104-106]

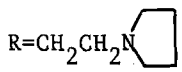
Miscellaneous Compounds - Centchroman (20) inhibited pregnancy in rats, mice, dogs and monkeys when administered once orally within 24 hours of coitus.[107] The drug was estrogenic, antiestrogenic and antiprogestational and inhibited ova development in the rat. Coadministration with P and estradiol diproprionate prevented the termination of pregnancy. Centchroman possessed both gonadotropin inhibitory and stimulatory properties in unilaterally ovariectomized rats depending upon the dose. The compound resembles in this respect the structurally related clomiphene (21), an antiestrogen and a known ovulation stimulator. Centchroman induced ovulation in women when administered daily.[108] The drug is well-tolerated[109] and is being studied as a once-a-week contraceptive.[110] The contraceptive activity was suggested to be a result of an inhibition of implantation and sperm transport.[111]

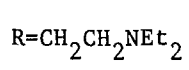


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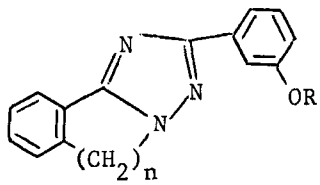


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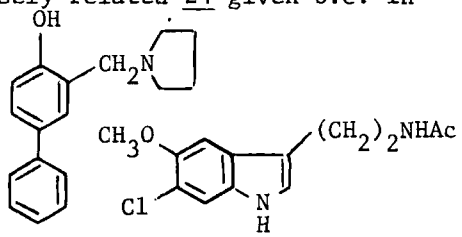
The triazoles 22 and 23, inhibitors of PG metabolism in the rat uterus, placenta, and lung,[112-114] were completely effective in terminating pregnancy in rats and hamsters when administered s.c. daily during any one of four 5-day periods between days 1 and 12 of pregnancy. These compounds were most effective when administered immediately following implantation; they lacked hormonal or antihormonal activity, and their antifertility effect was not reversed by P.[112] The closely related 24 given s.c. in



22 n=1,R=CH_3

23 n=2,R=CH_3

24 n=2,R=C_2H_5



25 26

combination with PGE_2 during estrus blocked ovulation almost completely in the hamster.[115] PGE_2 alone was ineffective and 24 alone was weakly active; 24 also hastened ova transport. Indomethacin, a PG synthetase inhibitor, suppressed spontaneous and induced ovulation in rats and rabbits,[116] suppressed gonadotropin-induced ovulation in rhesus monkeys[117] and marmosets,[118] but failed to inhibit spontaneous ovulation in rhesus monkeys.[119] Studies with indomethacin as an ovulation inhibitor in women were inconclusive[120] and in two small studies in women aspirin was ineffective.[121,122] The biphenyl derivative 25 was completely effective in inhibiting pregnancy in rats on days 4 and 5 at a dose of 5 mg/kg p.o.[123] 6-Chloromelatonin (26) inhibited ovulation in rats when administered i.v. or p.o. on the day of proestrus and was more effective than melatonin, probably as a result of an increased metabolic stability.[124]

A review of adrenergic innervation of the mammalian oviduct includes a summary of drugs that modify the sympathetic nervous system and affect ovum transport and fertility.[125] Plants are a relatively untapped potential source of new antifertility agents.[126]

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Chapter 17. Prostacyclin, Thromboxanes and the Arachidonic Acid Cascade

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Introduction - The discoveries of thromboxane A_2 (TXA_2) by Samuelsson's group in 1975[1] and of prostacyclin (PGI_2) by Vane's group in 1976[2] still remain the most recent and exciting breakthroughs in the prostaglandin (PG) area. With these new members, the arachidonic acid (AA) cascade (Fig. I) appears more complete and its physiological role is better understood and appreciated. Thus, TXA_2 generated by platelets promotes aggregation and constricts smooth muscle while PGI_2 produced by vascular endothelium, as well as by several other tissues, inhibits aggregation and relaxes smooth muscle. The biologically opposing effects of these substances apparently are of crucial importance to normal health and an inbalance in their production may lead to disorders such as abnormal blood pressure, atherosclerosis, stroke and heart attack. Therefore, the synthesis of these compounds and the search for more stable prostacyclins and thromboxanes become highly desirable not only to provide useful biological tools, but also compounds of therapeutic potential. The search focuses mainly on stable and selective biological agonists or antagonists of the natural molecules, as well as selective inhibitors of the various enzymes involved in their biosynthesis. The main scope of this article is to review the recent progress in the synthesis and biology of prostacyclins and thromboxanes. A review on the synthesis and biological

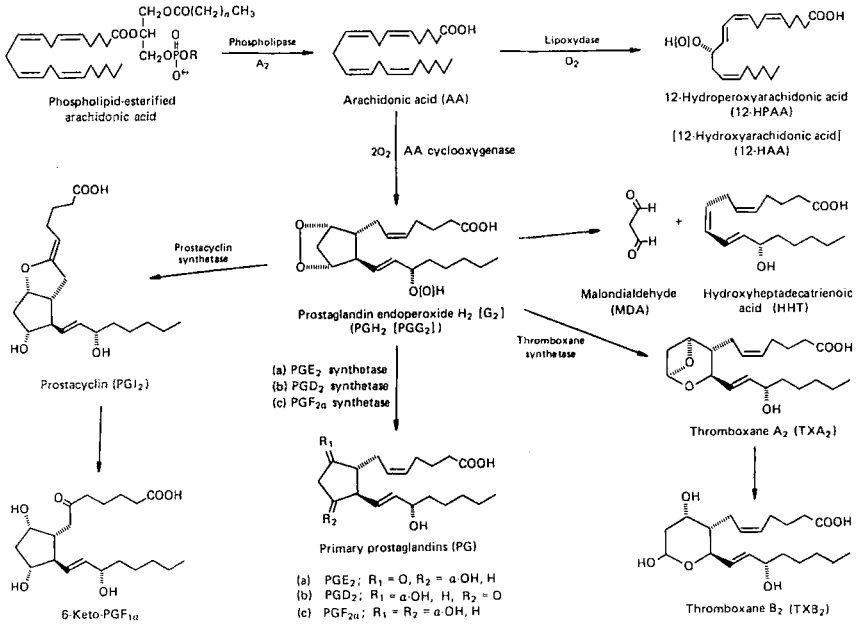
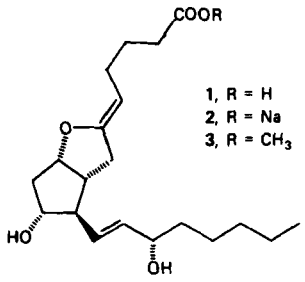


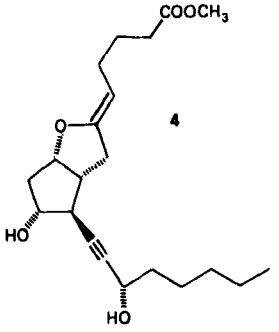
Figure 1. The arachidonic acid peroxidation pathways.

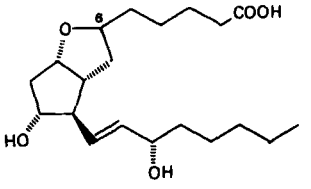
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properties of PG endoperoxides, thromboxanes and prostacyclins has recently appeared.[3] The last PG review in the Annual Reports in Medicinal Chemistry appeared in 1977.[4]

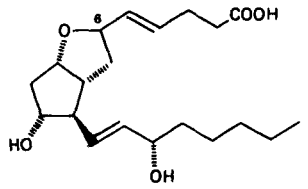
Synthesis of Prostacyclins - PGI_2 (1), its sodium salt (2) and methyl ester (3) have been efficiently synthesized from $PGF_{2\alpha}$ systems.[5-10] Unlike 1 and 3, the sodium salt 2, which is biologically equivalent to PGI_2, is stable both as a solid and in solution. The 13,14-dihydroprostacyclin methyl ester 4 was also synthesized and found equipotent to PGI_2 methyl ester as an inhibitor of platelet aggregation.[11] The stable prostacyclins 5a[5,6], 5b[5,6], 6a[5,12] and 6b[5,12] were synthesized, but showed much less potency than PGI_2 as inhibitors of blood platelet aggregation. The Δ^6 isomer (7) of PGI_2 has been reported to be eleven times more potent than PGE_1 (less potent than PGI_2) as an inhibitor of platelet aggregation.[13] The Δ^7 isomers 8a and 8b were synthesized[14] and proven to be different from the "Pace-Asciak and Wolfe PG"[15], thus requiring a revision of the structure of this compound. Sih has suggested the structure 7 for the "Pace-Asciak and Wolfe PG",[16] but ^{1}H NMR evidence strongly suggests that this PG possesses the ketal structure 9[10,13,17], which is obtained from



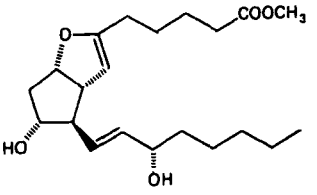




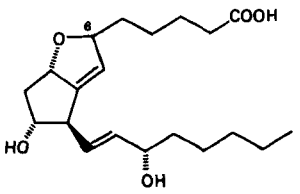
5a (6α)
5b (6β)



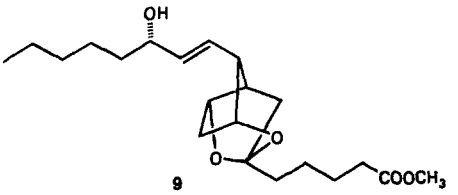
6a (6α)
6b (6β)



7

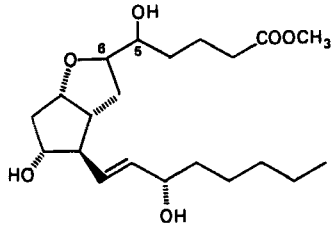


8a (6α)
8b (6β)

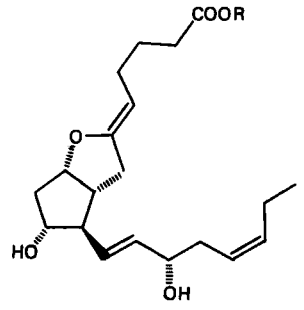


9

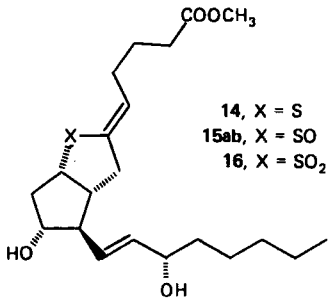
PGI_2 methyl ester under anhydrous acidic conditions. To test the intermediacy of 5-hydroxy-PGI_1 in the biosynthesis of PGI_2 all four isomers 10a-10d were synthesized[18,19] and subjected to prostacyclin synthetase.[18] None of these compounds, however, was converted to PGI_2 by the enzyme proving their non-involvement in the biosynthetic pathway.[18] PGI_3 (13) has been synthesized and found to possess similar biological properties as PGI_2.[6b,20] Sulfur-containing prostacyclins 14,[21,22] 15-17,[23] and 18-20[24] have been synthesized and found to be considerably more stable than PGI_2. The most extensively studied analog of this series, 6,9-thiaprostacyclin (14), showed 0.2-0.5 times the potency of PGI_2 as an inhibitor of blood platelet aggregation, but was a constrictor of the isolated cat coronary artery rather than a dilator.[21] The carbocyclic prostacyclin 21 and three of its isomers (C-5 and C-15) were synthesized recently.[25-27]



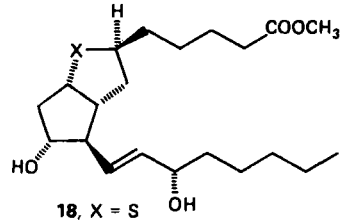
10a–d



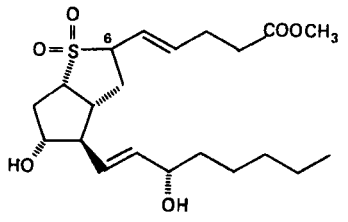
13



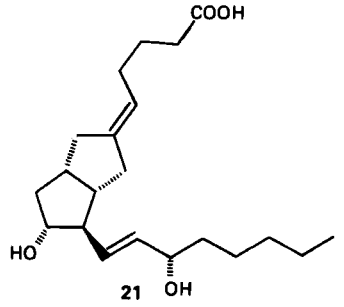
14, X = S
15ab, X = SO
16, X = SO_2



18, X = S
19ab, X = SO
20, X = SO_2

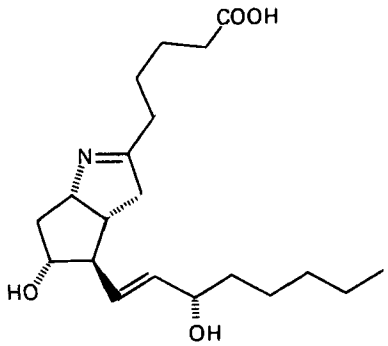


17a (6*S*)
17b (6*R*)

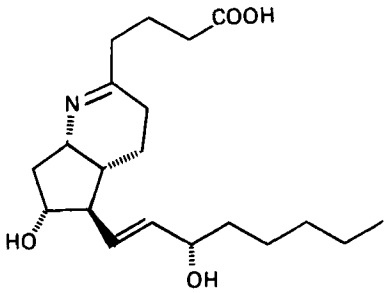


21

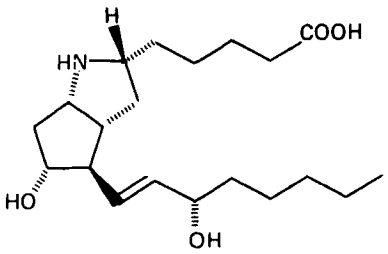
Isomer 21 exhibited higher potency than PGE_1, but lower than PGI_2 as an inhibitor or platelet aggregation and as a dilator of smooth muschle.[25,27] The N-containing prostacyclins (22-25) have also been reported and compounds 22 and 23 are surprisingly stable and potent biological mimics of PGI_2.[28] 6,9-Pyridazaprostacyclin (26) and a number of its derivatives have been synthesized from 6-keto-PGE_1 (27).[29] Both 26 and 27 showed higher potency than PGE_1 as inhibitors of platelet aggregation.[29] This first "aromatic" prostacyclin (26) was also found to be a potent dilator of the isolated cat coronary artery.[29] The 12-fluoro[30]-and 14-fluoroprostacyclins[31] have been synthesized and found to be potent agonists of PGI_2.[30,31]



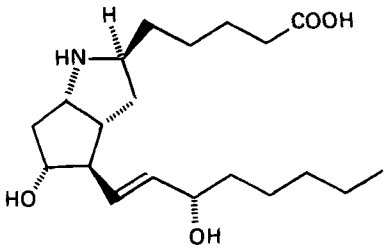
22



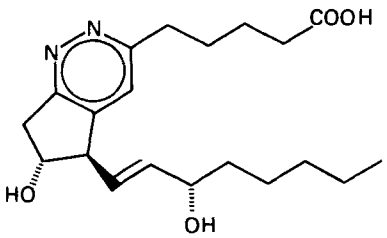
23



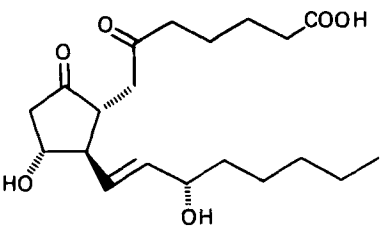
24



25



26

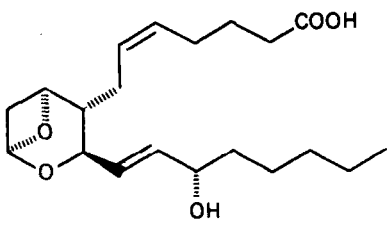


27

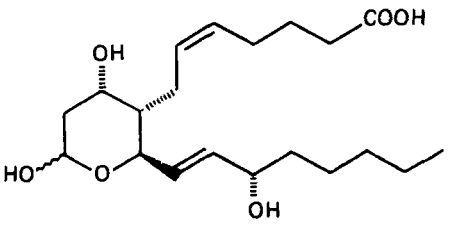
Synthesis of Thromboxanes - TXA_2 (28)[1] still remains elusive to isolation and chemical synthesis. Thromboxane B_2 (TXB_2) (29)[1], however, with its more accessible structure has been synthesized by several groups and is readily available.[32-38]

The first TXA_2 analog, pinane thromboxane A_2 (PTA_2) (30), has recently been synthesized in optically active form.[39] Unlike the 15-deoxy PGH_2 analogs 31[40] and 32[41], which are inhibitors of both thromboxane and prostacyclin synthetase, PTA_2 shows remarkable activity as a selective inhibitor of thromboxane synthetase without interfering with cyclooxygenase or prostacyclin synthetase.[39] Furthermore, it is a potent antagonist of TXA_2 and various stable endoperoxide analogs with regard to platelet aggregation and smooth muscle contraction.[39]

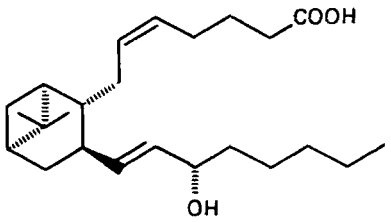
The methyl ester of PGH_2 (33) has been chemically synthesized[42] and so has[43] 12-hydroxyarachidonic acid (34).



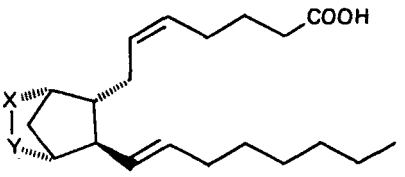
28



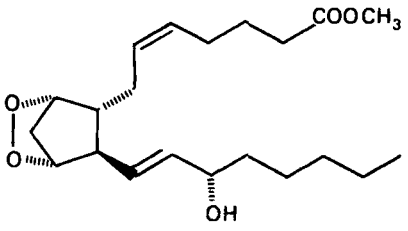
29



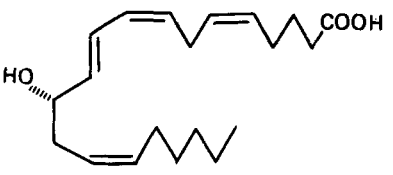
30



31, X–Y = N=N
32, X–Y = HN–O



33



34

Biosynthesis - PG endoperoxide synthetase has been purified to homogenity from the solubilized microsomes of vesicular glands[44-48] and has been partially purified from solubilized microsomes of platelets[49] and kidney medulla.[50] Its molecular weight is approximately 125,000 daltons based on the Stokes radius and sedimentation coefficient and it behaves as a single polypeptide of 69,000 - 85,000 daltons on sodium dodecylsulphate polyacamide gel electrophoresis indicating two subunits of approximately equal size.[44,47] The enzyme catalyses both cyclooxygenase ($AA \rightarrow PGG_2$) and peroxidase reactions ($PGG_2 \rightarrow PGH_2$) and requires hemin or a similar metalloprotein which is possibly lost during purification.[47,48] The peroxidase reaction also requires phenol or a related aromatic compound (e.g. tryptophan, serotonin, epinephrine, hydroquinone) which may act to supply electrons. These compounds also protect the enzyme from self-destruction by hydroperoxides such as H_2O_2[47] or PGG_2.[51]

PG endoperoxide E isomerase has been partially purified by solubilization of microsomes from bovine vesicular gland.[52] A sulphydryl-containing compound (e.g. glutathione, dithiothreitol, 2-mercapto ethanol) is required to stabilize the enzyme and glutathione acts specifically as a co-factor for the isomerization of PGH to PGE. The pathway PGG→15-hydroperoxy PGE→PGE originally suggested[53,54] seems unlikely since a) PGH rather than PGG is the preferred substrate, b) the isomerase does not contain peroxidase activity and converts PGG only to 15-hydroperoxy PGE and c) 15-hydroperoxy - PGE is not a substrate for purified PG endoperoxide synthetase and might well inactivate this enzyme as do other hydroperoxides.[47,51]

Thromboxane synthetase has been solubilized and partially purified from human[49,55] and bovine[56] blood platelets and from sheep[57] and bovine[58] lung. The enzyme activity also has been studied in platelet membranes by several workers.[59-63] Recent studies indicate that the same enzyme catalyses the formation of TXA_2 and hydroxyheptadecatrienoic acid (HHT) in a bimolecular reaction and that the formation of both products is inhibited in parallel.[54,60,61] These studies also show that the formation of HHT does not involve TXA_2 as an intermediate and that TXA_2 is converted exclusively into TXB_2.[2] PGH_3, the endoperoxide from eicosapentaenoic acid ($C_{20:5,5,8,11,14,17}$) is converted into thromboxane A_3 (TXA_3) by platelet membranes. Although TXA_3 contracts rabbit aorta, as does TXA_2, it does not induce platelet aggregation.[64] Eicosatrienoic acid ($C_{20:3,5,8,11}$) and its endoperoxide PGH_1, do not induce platelet aggregation as do AA or PGH_2.[64-67] Eicosatrienoic acid and PGH_1 are converted only in low yield to hydroxyheptadecadienoic acid and hardly at all into thromboxane A_1[61,65,68,69]. Neither 2-nor-PGH_2 nor 2-nor-PGH_1 are substrates for thromboxane synthetase[70] although 2-nor-PGH_2 induces platelet aggregation.

Pig aorta microsomes[2,71] and bovine coronary arteries[72,74] efficiently convert PGH_2 via PGI_2 into 6-keto-$PGF_{1\alpha}$.[6] The conversion of AA into 6-keto-$PGF_{1\alpha}$ has also been observed with microsomes obtained from ram[75] and bovine[76] seminal glands and from sheep lung.[77] Prostacyclin biosynthesis is highest in the ductus arteriosus, aorta and pulmonary artery of the fetal lamb[78] and has been detected in the cortex of rabbit and human kidney.[79] PGI_2 or 6-keto-$PGF_{1\alpha}$ has been detected in perfusates of rabbit and rat heart[80,81] and rabbit and dog lung.[82,83] The intima of vessel walls has the highest PGI_2 synthetase activity[84] and it is greater

in rat arteries than veins.[85]

There has been active, although not always consistent progress in studying PG synthesis by cells in culture. While mouse macrophages were found to synthesize PGE_2 and 6-keto-$PGF_{1\alpha}$ in response to zymosan,[86] activated macrophages from rats and guinea pigs were shown to produce TXB_2.[87] Cultured fibroblasts and smooth muscle cells were found to produce PGI_2 by some researchers[85,89] but had little PGI_2 synthetase activity when studied by others.[90-92] One possible explanation for the discrepancies is that the cultures of macrophages, fibroblasts and smooth muscle cells which produced PGI_2 were contaminated by endothelial cells. A consistent finding has been that endothelial cells synthesize PGI_2.[91-97] Collecting tubules from the rabbit renal papillae synthesize 6-keto-$PGF_{1\alpha}$ from AA.[98]

Inhibition of Biosynthesis - Inhibition of thromboxane synthetase has received a great deal of attention and a number of inhibitors of this enzyme have been reported. They include substrate analogs such as 9,11-epoxymethano-15-hydroxyprosta-5,13-dienoic acid[55,59], 9,11-azo-15-hydroxyprosta-5,13-dienoic acid[55,61], 9,11-epoxymethanoprosta-5,13-dienoic acid[59], 9,11-azoprosta-5,13-dienoic acid[40,99,100], imidazole[99-103] and some 1-substituted imidazole derivatives.[103] Whereas imidazole and its derivatives are weak inhibitors of platelet aggregation[99,101,103], 9,11-azoprosta-5,13-dienoic acid is a strong inhibitor.[99] It has been suggested that imidazole is only a weak inhibitor of aggregation because it facilitates the movement of intracellular calcium.[99]

Elaboration of the discovery that 15-hydroperoxy-AA inhibits PGI_2 synthetase[104] has revealed that 12-hydroperoxy-AA inhibits thromboxane synthetase[49], while ω-8 and ω-10 hydroperoxides of linoleic, dihomo-γ-linolenic and AA inhibit prostacyclin synthetase.[71] It seems possible that these hydroperoxides may perform a negative feedback role to inhibit excess thromboxane or PGI_2 formation. The effects of the 5-hydroperoxy-AA formed by rabbit polymorphonuclear leucocytes have not been investigated.[105]

Metabolism - TXB_2 is a poor substrate for the dehydrogenase which acts on PGE_2 and $PGF_{2\alpha}$, and TXB_2 remains the dominating compound in the circulation after its injection.[105] The major urinary metabolite of TXB_2 in the monkey is di-nor-TXB_2. Other metabolites, which result from changes in the 6-membered thromboxane ring and β-and ω-oxidation, have been identified.[107,108]

PGI_2 is rapidly oxidized in *vitro* by the 15-hydroxy-prostaglandin dehydrogenase of lungs[109] and blood vessels.[110,111] However, it is not a substrate for the lung transport system[109] and is not metabolized by intact lungs.[112] The major inactivation of PGI_2 appears to occur in the liver and hind quarters.[112] On the other hand, 6-keto-$PGF_{1\alpha}$ is a poor substrate for 15-hydroxy-PG dehydrogenase both in *vitro* and in *vivo*.[109,113] Seven urinary metabolites of PGI_2 including the di-nor and 19-hydroxy-di-nor derivatives of 6-keto-$PGF_{1\alpha}$ and 13,14-dihydro-6,15-diketo-$PGF_{1\alpha}$ and ω-carboxyl di-nor derivatives of 13,14-dihydro-6,15-diketo-$PGF_{1\alpha}$, have been identified.[114]

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Chapter 18. Drug Metabolism

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Introduction - Drug metabolism studies of current drugs may provide valuable clues for the design of new, safer drugs with greater specificity of action. This chapter will present selected examples of recent developments in the field of drug metabolism which may be helpful to the medicinal chemist and may provide insight into the current state of theoretical understanding and technological advances in the field.

Drug design must not only take into account the chemical and physicochemical properties which relate to intrinsic drug potency, but also those properties which relate to the ability of molecules to reach, and remain at, the site(s) of action in vivo. Clearly, properties such as rate and extent of absorption, distribution, biotransformation, and excretion have to be considered in drug design.

In the past few years the role of metabolic activation in the biogenesis of highly toxic entities has become increasingly clear. A knowledge of which biotransformation pathways tend to produce highly toxic metabolites, and which functional groups or molecular fragments are the best substrates for such bioactivation processes, may be used in the design of drug molecules for better safety as well as for the desired efficacy.

Recent Books and Reviews - Recently published books contain general information of drug metabolism in mammals[1] and in man,[2] drug disposition during development,[3] biotransformations with emphasis on those aspects which relate to the formation of toxic metabolites,[4-7] the disposition of toxic drugs in man,[8] bioavailability,[9] and methods and techniques related to drug disposition.[10,11] Other relevant books discuss drug design as related to adverse reactions,[12] prodrugs,[13] and sustained and controlled release.[14]

The following reviews are also of interest: "Effects of Renal Disease upon Drug Disposition,"[15] "Drug Metabolism and Pharmacokinetics in Malnutrition,"[16] "Novel Pathways in Drug Metabolism,"[17] "Individual Differences in the Disposition of Drugs Metabolized in the Body,"[18] "pK_a Values of Medicinal Compounds in Pharmacy Practice,"[19] "Metabolism of Drugs and Other Foreign Compounds by Intestinal Microorganisms,"[20] "Biliary Excretion of Drugs and Other Xenobiotics,"[21] "Drug-Induced Liver Disease,"[22] "Pulmonary Disease and Drug Kinetics,"[23] "Some Aspects of Pharmacokinetic and Biotransformation Differences in Human and Mammal Animals,"[24] "Disease and Drug Protein Binding,"[25] and "Toxic Agents Resulting from the Oxidative Metabolism of Steroid Hormones and Drugs."[26]

Methodology - Recently computers have been used by Wipke and coworkers to predict drug biotransformation pathways and products.[27] Pang and Gillette have published a series of equations for evaluating sites and pathways of metabolism from blood level data.[28] A method for determining binding constants for drug-protein complexes has been reported which utilizes serum albumin immobilized in microparticles of polyacrylamide.[29]

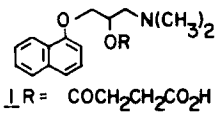
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Much of the recent development in gas-liquid chromatography (glc) has been in the use of more specific and sensitive detectors.[30-34] Separation of diasterioisomers of β-adrenoceptor antagonists (such as pindolol and propranolol) has been accomplished using high resolution capillary gas chromatography.[35] Radiochemical glc has been used in the analysis of ^{3}H-ethinyl estradiol in human urine.[36] Use of high performance liquid chromatography (hplc) is increasing. An hplc assay of procainamide was developed which requires as little as 20 µl of plasma.[37] Drug analysis by combined hplc-mass spectrometry has been reported by Henion.[38] A new thin-layer chromatography (tlc) chamber for short bed continuous development is now on the market.[39] A spectrodensitometric tlc assay has been reported for propranolol,[40] and a fluorescence tlc-densitometric assay has been developed for chlordiazepoxide.[41]

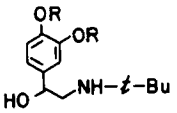
Prodrugs - Notari[42] has reviewed the potential utility of prodrugs which exhibit rate-limiting first-order drug delivery. The hemisuccinate ester 1 has been shown to provide protection against first-pass metabolism after oral administration, and has been proposed as a prodrug of propranolol (2).[43] The prodrug, bitolterol (3), the di-p-toluate ester of N-t-butylarterenol (4) has been shown to provide longer duration of bronchodilator activity in dogs than does the parent drug.[44]

Two prodrugs of the analgesic acetaminophen (5), the tetrahydropyranyl ether derivative (6) and the ethyl vinyl ether derivative (7), have been developed to overcome the bitter taste of the compound in chewable dosage forms.[45]

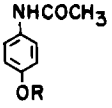
The compound, 7,7'-succinyl ditheophylline, was found to be a useful controlled-release prodrug of theophylline.[46] The feasibility of delivering highly polar drugs to the CNS was demonstrated using 9 as a prodrug for the pyridinium salt 8, which can cross the blood-brain barrier.[47] Prolonged antifertility activity for several contraceptive steroids was achieved in animals by subcutaneous injection of sustained release prodrugs in the form of zinc and aluminum tannate salts of tertiary amine derivatives.[48]



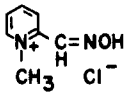
1 R = $COCH_2CH_2CO_2H$
2 R = H



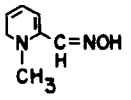
3 R = p-Toluoyl
4 R = H



5 R = H
6 R = THP
7 R = $CH(CH_3)OCH_2CH_3$



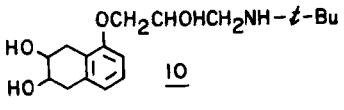
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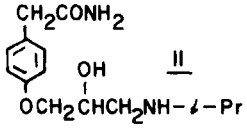
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Pharmacokinetics and Pharmacodynamics - A good correlation was shown between the logarithm of blood concentration and the percentage reduction in exercise heart rate for propranolol (2) indicating, for this drug, predictability of β-adrenoceptor blockade from blood concentrations.[49] A dose-response relationship was shown between blood levels of *d*-amphetamine and magnitude of blood pressure and heart rate responses in rats under ganglionic blockage.[50] The dose-dependent (nonlinear) pharmacokinetics of drugs may have important clinical implications. A disproportionate increase in steady-state plasma concentrations of quinidine was observed in man with increasing doses.[51] Comparing the values of area under the curves of plasma concentration vs. time following oral and intravenous administration of the antiarrhythmic drug disopyramide led to an erroneous estimate of its bioavailability in man, since clearance of the drug was different for the two routes of administration.[52] Nonlinear elimination kinetics of sulfadiazine in rabbits were attributed to protein-binding interactions between the parent compound and its N-acetyl metabolite.[53]

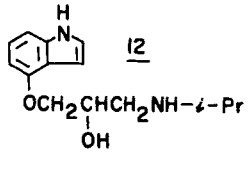
A linear relationship was observed between the renal clearance of nadolol (10) and the glomerular filtration rate (GFR) in dogs in different stages of experimentally-induced renal impairment.[54] The body clearance of two other β-adrenergic blocking agents, atenolol (11)[55] and pindolol (12),[56] was also found to correlate with the GFR. Reduction in bioavailability of pindolol in renally impaired patients was attributed to a decrease in drug absorption.[56] The rate of elimination of netilmicin, an aminoglycoside, was found to decrease proportionally with decreasing renal function.[57] Pharmacokinetic studies of Lidaprim® (a trimethoprime-sulfametrole combination) in renally impaired patients showed a strong influence of renal function on the accumulation of the metabolized sulfonamide, a weaker influence on trimethoprime, and negligible influence on the free sulfonamide.[58]



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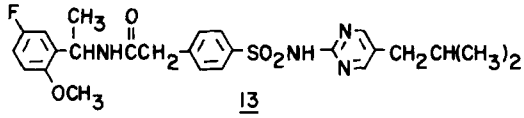
12

The renal clearance of sulfamethoxazole, but not of its metabolite, N_4-acetylsulfamethoxazole, was markedly influenced by urinary pH and urine flow.[59] Pharmacokinetic analysis of percutaneous absorption showed evidence of parallel penetration pathways for methotrexate when it was topically applied to hairless mouse skin.[60]

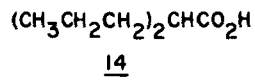
A recent review summarized the saliva to plasma ratios of a number of drugs and discussed the usefulness of salivary concentrations in therapeutic drug monitoring and in calculation of pharmacokinetic parameters.[61] Interrelationships among renal hemodynamics, drug kinetics and drug action was recently reviewed.[62] The effect of pulmonary disease on disposition kinetics of drugs has been reviewed.[23] A mini-review on pharmacokinetic and biotransformation differences among mammalian species suggested that species differences are usually of a quantitative nature, due to such differences as the ratio of body weight to surface area, organ weight

to body weight, or presence of differing fat deposits.[24]

Plasma and Tissue Protein Binding - The extent of, and changes in, the binding of drugs and their metabolites to plasma and tissue proteins can greatly affect their distribution and elimination and may, therefore, influence the extent and duration of effect. A comprehensive review of plasma protein binding of drugs in disease states and its influence on the pharmacologic effects was recently published.[25] The R (+) enantiomer of gliflumide (13), a new antidiabetic drug, was less potent in lowering blood glucose in rats than gliflumide and had reduced affinity to plasma protein, indicating the binding to receptor sites and to plasma protein was stereospecific.[63] Protein binding studies of a new cholangiographic agent, iodoxamic acid, to monkey plasma indicated two classes of binding sites, and binding solely to plasma albumin.[64] Serum protein binding studies in man, dog, rat, and mouse of the anticonvulsant drug valproate (14) indicated that increased drug binding was associated with a decrease in the total body clearance of the drug.[65] The effect of protein binding on pharmacokinetics and therapeutic activity of several cephalosporin antibiotics was recently reviewed.[66] Theoretical relationships were presented for the assessment of changes in the binding of drugs to tissues, based on determinations of total clearance, intrinsic clearance, apparent volume of distribution, and biological half-life.[67]



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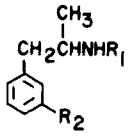
Drug Interactions - Absorption half-life of the antibiotic, clindamycin, was prolonged in human subjects when a kaolin-pectin antidiarrheal suspension was coadministered, although the extent of absorption remained the same.[68] On the other hand, both the rate and the extent of absorption of digoxin were lower when it was coadministered with the same kaolin-pectin suspension.[69] Physical absorption and alterations in gut transit time appeared to partly explain the findings.

The antitussive activity of dextromethorphan hydrobromide in the unanesthetized dog was faster in onset, greater in intensity, and longer in duration when it was coadministered with salicylamide and acetominophen, due probably to inhibition of the metabolism (conjugation) of dextrorphan, an active metabolite of the parent drug.[70] In rats, acute administration of ethanol increased the concentrations of methadone in brain and liver, apparently due to inhibition of methadone metabolism in the liver, whereas chronic ethanol administration had the opposite effect due to increased liver microsomal metabolism of methadone.[71] In rat liver preparations, glucuronidation of acetaminophen was inhibited by a variety of drugs, e.g., morphine, dicumarol, hydroxyzine, phenolphthalein, chloramphenicol, and tetracycline.[72] In dogs, indomethacin pretreatment inhibited the hemodynamic response to furosemide and reduced the renal and extrarenal clearance of furosemide by approximately 30%, but did not change the proportion of unchanged drug excreted in urine.[73] The effects of alterations in blood flow, enzyme activity, and reversible drug binding to blood components on total body clearance have been discussed.[74]

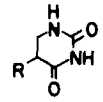
Drug Metabolizing Enzymes - The possible role of excited states of oxygen (e.g., singlet oxygen and the superoxide ion) in cytochrome P-450 linked oxidations, as well as in the induction of the P-450 system by many diverse compounds, has been discussed.[75] A good correlation was found between the binding affinity of a wide variety of organic compounds to cytochrome P-450 and their lipophilicity.[76] The enzyme system responsible for 7 α-hydroxylation of cholesterol was found to be distinct from the mixed function oxygenases system that catalyzes oxidation of a large number of xenobiotics.[77] In rabbit liver, two stereoselective enzyme systems, or different conformations of binding of different substrates to the same enzyme system, were suggested to be responsible for α-carbon oxidation and N-oxidation of ethylamphetamine (15).[78] Chromatographic and electrophoretic studies indicated extensive heterogeneity of cytochrome P-450 from rat liver.[79]

Dihydropyrimidinase, an enzyme responsible for the hydrolytic ring opening reactions of dihydrothymine (16) and dihydrouracil (17), was partially purified and characterized from calf and rat liver. The enzyme from both sources catalyzed the hydrolytic ring-opening of dihydrouracil, hydantoin (18), 5-phenylhydantoin (19), and α-phenylsuccinimide (20).[80]

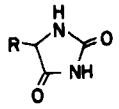
Because many drugs contain either chiral centers, prechiral centers, or both, interest in stereochemical substrate-enzyme interactions, the stereospecificity of biotransformations, and species (and strain) differences in these parameters is increasing. Since enzymes themselves contain chiral centers, differential interaction of R and S isomers of drugs with drug metabolizing enzymes is the rule rather than the exception. Beckett[81] reported stereoselectivity in the N-dealkylation, deamination, and formation of the nitrone and secondary hydroxylamine metabolites (+) - and (-) - N-benzylamphetamine (21) in rabbits. Stereoselectivity has also been observed in the dealkylation of d-, l-, and d,l-fenfluramine (22), an anorexiogenic agent.[82]



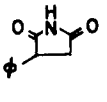
15 $R_1 = CH_2CH_3$, $R_2 = H$
21 $R_1 = CH_2\phi$, $R_2 = H$
22 $R_1 = CH_2CH_3$, $R_2 = CF_3$



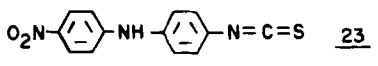
16 R = CH_3
17 R = H



18 R = H
19 R = ϕ



20

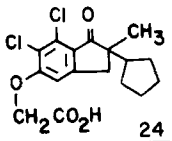


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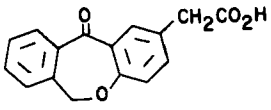
Extrahepatic Drug Biotransformation - Although the liver is the major organ for biotransformation of xenobiotics, other organs, e.g., kidney, lung, intestine, etc., also possess this capability to varying degrees. Glucuronidation of diethylstilbestrol was shown to occur in all regions

of the intestine.[83] Both glucuronidation of a major digitoxin metabolite and hydrolysis of the glucuronide conjugate took place in various segments of the rat intestine.[84] The effect of gut wall metabolism, hepatic elimination and enterohepatic recycling on estimates of bioavailability have been examined from a theoretical standpoint.[85] An antischistosomal isothiocyanate (23) was converted to a mutagen in six mammalian species by intestinal bacteria (metabolic activation).[86] Oxidative metabolism of polycyclic aromatic hydrocarbons to arene oxides (epoxides) was observed in the lung.[87] In the isolated perfused rabbit lung, Δ^9-tetrahydrocannabinol was metabolized to at least six metabolites.[88]

Interspecies Comparative Metabolism - An unusual species difference was reported for absorption of nadolol[89] (10). Whereas, absorption of an oral dose of nadolol in mice, rats, hamsters, rabbits, monkeys, and man ranged from ca. 8 to 34%, the absorption in the dog was essentially complete. Species differences in metabolism appeared to explain in part the inconsistent and low physiologic response (diuresis) of MK-473 (24) observed in the dog, a species in which minimal MK-473 metabolism was observed.[90] In other species, a number of pharmacologically active diestereoisomeric metabolites were produced by hydroxylation of the cyclopentyl ring. Reduction of the 6-keto group in hydrocodone to the β-alcohol was stereoselective in various animal species, but not in man.[91] In the rabbit and dog, the principal metabolites of isoxepac (25) were the glycine and taurine conjugates, respectively, whereas in the rhesus monkey and man, isoxepac was excreted unchanged or as the glucuronide.[92]



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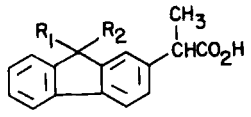


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Biotransformation and Drug Toxicity - In the past few years significant progress has been made in understanding the relationship between biotransformation and toxicity of numerous drugs. Evidence has been presented that biotransformation is responsible for the unusual triphasic lethal dose curve obtained for *d*-amphetamine in mice.[93] The *in vitro* conversion of the N-O-glucuronide and N-O-sulfate conjugates of N-hydroxyphenacetin to reactive intermediates has been reported.[94] Isolation of 2-hydroxyphenacetin as a decomposition product also suggested a possible non-arene oxide pathway for the aromatic hydroxylation of certain aromatic amines. Metabolic activation is used routinely in mutagenicity testing, and a method has recently been presented for using metabolic activation in assessing cytotoxicity.[95] Awareness of the greater potential for certain molecular substructures to result in carcinogenicity of drugs containing them has led the FDA's Bureau of Foods to publish a list of 13 substructural moieties which enhance the probability of carcinogenicity.[96] Alteration of drug biotransformation by structural modification to reduce toxicity has been discussed,[13] as have the problems in relating toxicological effects and drug disposition.[97] A dose dependency for acetaminophen-induced renal necrosis in rats has been demonstrated.[98] Differences in the mutagenicity of the 7,8- and 9,10-dihydrodiols of benzo[a]pyrene have

been attributed to differences in metabolic pathways.[99]

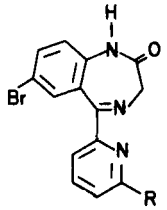
Novel Biotransformation Pathways - The recent literature contains examples of some of the newly discovered pathways in addition to those covered in the excellent review by Jenner and Testa.[17] Formation of an aliphatic O-methyl metabolite (26) of the antiinflammatory agent cicloprofen (27) in the rat, has been demonstrated,[100] and it was proposed that it formed via O-methylation of a dihydroxy intermediate (28). Thiomethyl metabolites of phenacetin and acetaminophen have been found in dogs and man.[101] An S-methyl metabolite (29) of bromazepam (30), a minor tranquilizer, in the rat was shown to be formed in vitro by biotransformation of a mecapturic acid conjugate (31).[102]



26 R_1 = OH, R_2 = OCH_3

27 R_1 = R_2 = H

28 R_1 = R_2 = OH



29 R = SCH_3

30 R = H

31 R = $SCH_2CH(CO_2H)NHCOCH_3$

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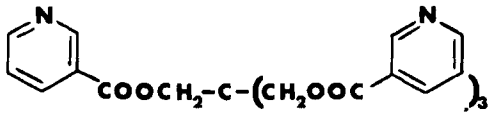
Chapter 19. Disorders of Lipid Metabolism

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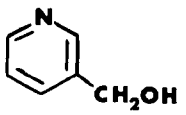
Introduction - The Lipid Hypothesis is the premise that reducing the levels of serum lipids in patients with hyperlipidemia will lead to a reduction in the incidence of coronary heart disease (CHD).[1] In last year's chapter of this series, the pharmacological control of serum lipoproteins was reviewed.[2] In this review, recent developments in atherosclerosis regression, high density lipoproteins, and established and newer antihyperlipidemic agents are outlined. Recent work on the metabolic disposition of some of the lipid lowering drugs is discussed. Finally, advances in the therapeutic control of cholesterol gallstones are summarized.

Regression of Atherosclerosis - The goal in establishing the various risk factors of CHD is to determine whether the course of the disease can be modified by adjustment of the individual's life style. Evidence has begun to accumulate that human atherosclerosis can be arrested or partially reversed.[3] Animal models used to study the reversal of experimental atherosclerosis have been recently reviewed.[3,4] Non-human primates, rabbits, swine, fowl and dogs are commonly used. The aims of animal studies are to explore the mechanisms underlying regression, and to establish treatment of the human disease by diet, drugs, surgery and/or exercise.[4]

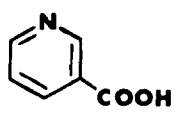
Recently, diet-induced regression has been demonstrated in swine[5] and monkeys.[6,7] Alfalfa meal counteracted the atherogenicity of dietary cholesterol in cynomolgus macaques.[8] Niceritrol (1), β-pyridylcarbinol (2) and, to a lesser degree, nicotinic acid (NA) (3) partially reversed diet induced atherosclerosis in minipigs.[9] A greater reduction in the size of coronary lesions in atherosclerotic minipigs was found with a combination of moderate diet plus clofibrate than with diet alone.[10] Cholestyramine produced a partial reversal of atherosclerosis in rhesus monkeys.[7] Clofibrate retarded the development of spontaneous atherosclerosis in repeatedly-bred Sprague-Dawley rats,[11] and reduced the morbidity and mortality from acute myocardial infarction induced by intravenously injected isoproterenol.[12] The antimetabolites mercaptopurine and hydroxyurea were anti-atherogenic in swine.[5] It was suggested that the anti-atherogenic action of pyridinolcarbamate and phthalazinol in rabbits and man was due to inhibition of platelet aggregation resulting from antagonistic effects on thromboxane-A_2.[5,13] On the other hand, the antihypertensive drugs hydrochlorothiazide, methyldopa, guanethidine, minoxidil or hydralazine did not alter the severity or extent of atherosclerotic lesions in stumptail macaques with hypertension (induced by narrowing of the renal artery) and atherosclerosis (high fat-cholesterol diet).[6]



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A decrease in serum cholesterol levels results in a decrease not only in the lipid content but also in the size of experimentally induced atherosclerotic lesions. Evidence of this association in man has been demonstrated by coronary angiography.[14] Human femoral atherosclerosis regressed in response to diet and/or drug therapy (clofibrate, NA, or clofibrate in combination with neomycin).[14,15] The degree of retardation of atherosclerosis was directly correlated with the decrease in serum cholesterol levels.[14,16] Regression in patients with Type IV hyperlipoproteinemia was also associated with the decrease in serum triglyceride levels;[16] however, no such correlation was found in another study.[15]

Serum lipids and lipoproteins - The study of the control of serum lipid levels and their relationship to CHD has evolved through several phases. Initially, the emphasis was on serum cholesterol levels, stimulated no doubt by the availability of methods to measure cholesterol. Epidemiological studies established cholesterol as a risk factor of CHD. This was followed by consideration of serum triglycerides, the role of which as an independent risk factor still remaining to be fully elucidated.[17] Later, it became apparent that examination of the control of lipid transport is necessary for the understanding and management of lipid disorders. As analytical methodology became more sophisticated, the role of individual lipoproteins in atherosclerosis began to be evaluated. Thus, low density lipoproteins (LDL), very low density lipoproteins (VLDL) and chylomicrons are atherogenic, while high density lipoproteins (HDL) are considered anti-atherogenic. All the lipoproteins are metabolically related and interact dynamically within the vascular system.

As clinical evidence continues to accumulate on the inverse correlation between HDL-cholesterol (HDL-C) levels and the incidence of CHD,[18-23] considerable attention is being devoted to the metabolic control of HDL and factors affecting circulating HDL levels (reviewed in refs. 24-30). Two mechanisms have been proposed (not necessarily mutually exclusive) whereby HDL may slow atherogenesis.[23,28] First, HDL may facilitate the removal of cholesterol from the arterial wall.[31] This action is mediated by the action of lecithin-cholesterol acyltransferase (LCAT), the enzyme which catalyzes the esterification of free cholesterol in the HDL polar surface. As the cholesterol esters migrate to the nonpolar core of HDL, the modified surface of HDL could accept more free cholesterol from the peripheral cells. The possible role of HDL in cholesterol excretion was suggested by a study which showed that cholesterol from HDL was more readily incorporated into biliary cholesterol than cholesterol from LDL.[32] Second, HDL may inhibit the uptake of atherogenic LDL by the arterial wall. The pioneering work of Goldstein and Brown has established the function of LDL receptors in the pathogenesis of atherosclerosis, [33] and in vitro models with various human and animal cell types have shown that HDL inhibits LDL binding and uptake by the cells.[28]

It remains to be established whether manipulation of HDL levels will in itself alter the development of CHD. The components of HDL are derived from different sources.[24,26,29,30,34] HDL (or its precursors) is produced by both the liver and intestine. The structural composition of HDL is regulated by LCAT and lipoprotein lipase. There is also non-enzymatic transfer of apolipoprotein (apo) C between HDL and VLDL, exchange of free

cholesterol between HDL and cell membranes, and the association of apo A-I with HDL. Part of circulating HDL is derived from catabolism of VLDL and chylomicrons, and numerous studies have shown reciprocal relationships between HDL-C and serum triglycerides.[26,29,35]

Agents which induce liver microsomes elevate HDL-C; thus alcohol consumption,[36] exposure to chlorinated hydrocarbon insecticides,[37] and treatment with phenytoin[38] caused increased HDL-C. HDL-C was elevated by exercise,[39] and in men treated with estrogen for prostatic carcinoma.[40] HDL-C was lowered by smoking,[41,42] dietary carbohydrate,[43] and by antihypertensive therapy with a combination of hydrochlorothiazide and propranolol.[44] Oral contraceptives were reported to both increase[45] and decrease[42] HDL-C. Since estrogens elevate and progestins lower HDL-C, the effect of oral contraceptives on HDL-C appears to depend upon the relative estrogen/progestin potencies of the preparations.[46]

Antihyperlipidemic agents - Clofibrate - A monograph on clofibrate has recently been published.[47] The recently completed WHO primary prevention trial showed that clofibrate treatment reduced the incidence of non-fatal myocardial infarcts by 25%, but did not alter the incidence of fatal heart attacks.[48] In most of the studies reported during the past year, HDL-C was significantly elevated by clofibrate.[49-52] Whether or not clofibrate elevates HDL-C may depend upon the pretreatment level. Thus, if the initial levels were below 40 mg/dl, clofibrate elevated HDL-C, e.g. from 30.2 to 44.1 mg/dl[49] or from 37.5 to 44.9 mg/dl.[52] On the other hand, in patients who had a myocardial infarction but relatively normal HDL-C, clofibrate did not elicit a statistically significant rise in HDL-C.[53] Clofibrate increased apo A-I by 11% and apo A-II by 27%, implying a preferential increase in HDL_3 (d = 1.125-1.210).[50]

Combination therapy with clofibrate and NA (or NA prodrugs)[49,54,55] or with colestipol[51,56] produced additive effects on serum lipids in properly selected patients. Thus, clofibrate enhanced the cholesterol lowering action of colestipol in subjects with Type IIa hyperlipoproteinemia, but the combination of the two drugs produced a net increase in serum cholesterol in Type IIb hyperlipoproteinemia.[56] A preliminary report of the ongoing Stockholm study[54] in survivors of myocardial infarction who were treated with both clofibrate and NA showed no effect on total mortality or on mortality from CHD, but a significant reduction in non-fatal myocardial infarctions was demonstrated. Combinations of clofibrate with a sulfomucopolysaccharide preparation,[57] phenformin[58] or etiroxate(α-methyl-DL-thyroxine ethyl ester)[59] lowered serum lipids to a greater extent than did clofibrate alone.

The mode of action of clofibrate has yet to be completely elucidated. Sterol balance data in man supported the widely accepted concept that the drug inhibits cholesterol biosynthesis.[60] However, in rats fed 0.25% clofibrate for 2 weeks, no inhibition in the absolute rates of cholesterol biosynthesis was observed in liver, intestinal wall or adipose tissue ($[^3H]_2O$ incorporation to cholesterol).[61] Intralipid clearance from the plasma of rats[61] and hyperlipidemic patients[62] was enhanced by clofibrate, demonstrating increased fractional turnover rate of endogenous triglyceride. Clofibrate enhanced the activity of post-heparin plasma lipo-

protein lipase activity in man, but had no effect on hepatic triglyceride lipase.[63,64] In addition, clofibrate increased the levels of adipose tissue lipoprotein lipase in human patients[62] but not in cholesterol-fed rats.[61] The drug decreased the mean molar LCAT rate by 10% in hypertriglyceridemic subjects whose initial LCAT rates were relatively high, but did not alter the mean fractional LCAT rate.[52]

It has long been known that clofibrate increases hepatic peroxisomes but whether a relationship exists between peroxisome proliferation and the hypolipidemic action of the drug has not been established. Several hypolipidemic clofibrate analogues did not proliferate hepatic microsomes in rats, thus suggesting that both effects occur independently.[65]

The metabolic disposition[66] and clinical pharmacokinetics[67] of clofibrate have been recently reviewed. In healthy volunteers, the plasma elimination half-life ($t_{\frac{1}{2}}$) of chlorophenoxyisobutyric acid (CPIB), the pharmacologically active form of clofibrate, averaged 17 hr, independent of dose (500-2000 mg) or duration of treatment.[68] The plasma clearance and apparent volume of distribution were identical for all doses when based on unbound CPIB concentrations, but clearance calculated from total CPIB levels increased with increasing dose; this was due to decreased protein binding at higher plasma concentrations.[68] In rats, the serum $t_{\frac{1}{2}}$ of CPIB was 5 hr, but a second exponential with longer $t_{\frac{1}{2}}$ occurred at later time intervals; the $t_{\frac{1}{2}}$ in dogs was 40-50 hr.[69] At 100 µg CPIB/ml serum, the percentage free CPIB was 25, 16 and 3% in rats, dogs and man, resp.[69] Co-administration of the anion exchange resins colestipol or cholestyramine with clofibrate to human subjects did not affect the bioavailability of CPIB.[70] In rats, clofibrate increased the area under the serum concentration/time curve of NA, and it was concluded that clofibrate decreased the rate of urinary excretion of NA.[71] Clofibrate did not modify the $t_{\frac{1}{2}}$ of antipyrine nor urinary glutaric acid excretion in normal subjects, thus the drug is not an enzyme inducer in man.[72]

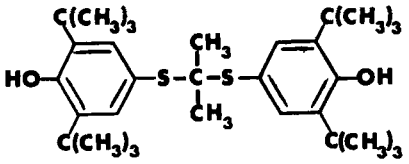
Nicotinic acid (NA) and derivatives - Patients with severe hypercholesterolemia were treated with 0.9-1.2 g/day of β-pyridylcarbinol (2) for up to 9 years; therapy reduced serum cholesterol levels, and no myocardial infarction was recorded in the 12 patients completing the study.[73] The antiatherogenic effects of NA, 2, and two other NA derivatives in rabbits were accompanied by an elevation of the HDL/LDL cholesterol ratio in the aortic tissue.[74]

The mode of action of NA[75] continues to be the subject of extensive investigation. When hyperlipidemic patients were given 4.5 g NA/day, there was an initial fall in lipids associated with both VLDL and HDL; after the first few days, HDL-C became elevated, independent of any change in VLDL-triglyceride concentration.[48] The same pattern was observed in patients given clofibrate (1.5 g/day) or clofibrate plus β-pyridylcarbinol (75 mg/day).[49] NA decreased cholesterol biosynthesis and increased the fractional turnover rate of plasma triglycerides, but had no effect on plasma lipoprotein lipase or LCAT activity; it was concluded that the various effects of NA were secondary to its antilipolytic action on adipose tissue.[76] NA did not alter biliary lipids in man, and therefore should not increase the risk of gallstone formation.[77] In vitro, NA inhibited thromboxane A_2 formation by phospholipase A_2 in rat platelets.[78]

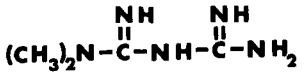
Apo C-II, the activator of lipoprotein lipase, was absent in a subject with severe hypertriglyceridemia; a transfusion of plasma produced a temporary fall in the patient's circulating triglyceride, due to apo C-II in the normal donor's plasma.[79] It was also reported that NA (12 g/day) increased apo C-II in chylomicrons and VLDL of a subject with Type V hyperlipoproteinemia.[80] These studies support the concept that low amounts of apo C-II may play a role in the development of hypertriglyceridemia.

Ion exchange resins - The bile acid sequestering anion exchange resins (cholestyramine, colestipol and polidexide) continue to show utility in lowering serum cholesterol levels in Type II hyperlipoproteinemia.[81-83] A large scale trial with patients treated for up to 3 years with colestipol showed lower cardiovascular mortality in men, but not in women.[83] The resins lower LDL-C; a study with colestipol showed no change in HDL-C.[50] The metabolic disposition of oral [^{14}C]colestipol was studied in rats, dogs and man; absorption was negligible and virtually all of the radioactivity was excreted in feces.[84]

Probucol - The pharmacologic and therapeutic properties of probucol (4) have been recently reviewed.[85] Probucol lowered plasma cholesterol levels without affecting triglyceride levels.[85,86] The mode of action of the drug is unknown. Pharmacokinetic studies in man[85] showed limited and variable absorption. Upon oral administration, steady state plasma levels were attained only after several months of treatment. Following long-term dosing, the plasma $t_{\frac{1}{2}}$ was several weeks. Tissue distribution studies in rhesus monkeys[85] showed substantially higher tissue than plasma levels; probucol was retained in adipose tissue.



4



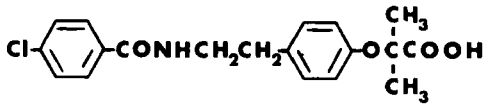
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β-Sitosterol - Recent reports demonstrated the efficacy of β-sitosterol in lowering plasma LDL-C in patients with Type II hyperlipoproteinemia.[87,88] β-Sitosterol obtained from tall oil was found to be effective at 3 g/day, a dose substantially lower than that required with older preparations.[87] However, in juvenile Type II hyperlipoproteinemia, β-sitosterol lowered LDL-C by only 6% but, in addition, decreased HDL-C by 15%; thus, β-sitosterol was not recommended for the treatment of hypercholesterolemia in children.[89] A potential problem with β-sitosterol is the increased risk of gallstone formation, in view of the finding of increased saturation index of bile in patients given 3 g/day of plant sterols.[90]

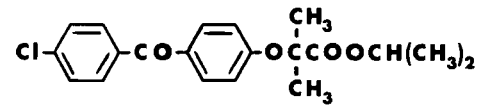
Metformin - The antiatherogenic action of the hypoglycemic biguanide metformin (5) appears to be associated more with alterations in apolipoprotein structure and composition than with lipid lowering effects.[91] Six-month therapy of 254 hyperlipidemic patients with 2.5 g/day of metformin produced an average reduction in serum triglycerides of 57%, but no change in cholesterol levels; metformin therapy improved various hemodynamic

parameters (e.g. blood pressure, EKG).[92]

Bezafibrate - Bezafibrate (6), an analogue of clofibrate, had similar effects on serum lipids and lipoproteins as did clofibrate, but at lower doses.[93] The pharmacologic spectrum of both drugs in rats was similar.[94,95] In man, bezafibrate lowered serum triglycerides; HDL-C increased by 31%, independently of a decrease in VLDL-triglyceride.[96] The degree of LDL-C decrease was dependent upon pretreatment levels.[96] The optimum dose was 450-600 mg/day, and serum lipoprotein changes were maintained during one year of therapy.[97] Bezafibrate lowered serum lipid levels in hyperlipidemic diabetics.[98] As measured by GLC,[99] bezafibrate was reported to be completely absorbed in man, and had a $t_{\frac{1}{2}}$ of 2 hr.[100] Despite the short $t_{\frac{1}{2}}$, the antihyperlipidemic activity lasted for 24 hr.[101]



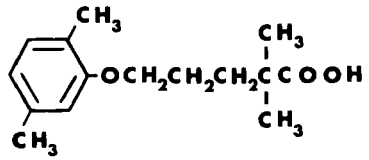
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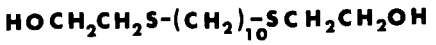
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Procetofene - In a 3-year trial in 340 patients with Types IIa, IIb and IV hyperlipoproteinemia, procetofene (7) produced dose-dependent (200-400 mg/day) decreases in serum cholesterol (20-36% reduction) in Type II patients and 20-50% decreases in serum triglycerides in Types IIb and IV subjects.[102] Colestipol plus procetofene produced greater reductions in cholesterol levels in Type II patients than did procetofene alone.[82] In a comparative study, LDL-C in Types IIa and IIb patients was lowered 37% by procetofene, 27% by bezafibrate and 18% by gemfibrozil (8).[104] The three drugs decreased VLDL-triglyceride levels by 45-58% in Type IV hyperlipoproteinemia.[104] In man, the $t_{\frac{1}{2}}$ of procetofenic acid, the active metabolite of procetofene, was 24 hr.[103]

Tiadenol - Tiadenol (9) (2.4 g/day) lowered serum cholesterol levels in Type IIa hyperlipoproteinemia and decreased serum triglycerides in Type IV patients; serum lipids were unaltered in Type IIb patients.[105] In vitro, tiadenol inhibited lipid mobilization as well as phosphodiesterase activity in rat epididymal fat pads.[106]



8

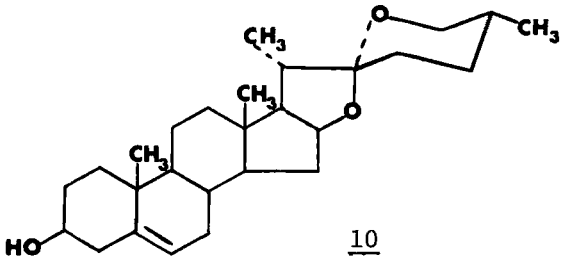


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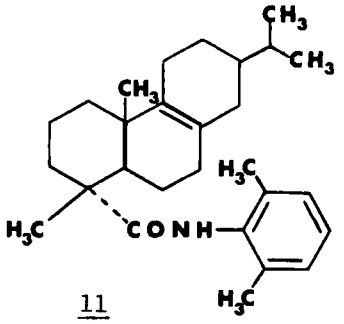
Estrogen - Women with Type III hyperlipoproteinemia were given 1 μg/kg/day of ethinyl estradiol; VLDL cholesterol, triglyceride and apolipoprotein composition became normal.[107] This was in contrast to the usual hypertriglyceridemic activity of estrogen in normal subjects, and it was postulated that, in the Type III syndrome, estrogen increased the rate of clearance of VLDL remnants from the plasma.[107] Postmenopausal women with Type II hyperlipoproteinemia were given 2 mg/day of an estradiol derivative for 6 months.[108] LDL-C declined by an average of 18% and HDL-C rose by 30%, while serum triglycerides were unaltered; the data demonstrate another indication for estrogen therapy in postmenopausal women.[108]

Sucrose polyester - In normal volunteers, doses of 8-25 g/day of sucrose polyester, a liquid non-hydrolyzable unabsorbable long chain fatty acid ester of sucrose, lowered total and LDL-C without altering serum triglycerides or HDL-C.[109] The material inhibited cholesterol absorption and increased the excretion of neutral sterols and bile acids.[110]

Sapogenins and saponins - Diosgenin (10) elevated HDL-C and lowered LDL-C in cholesterol-fed rats; the agent inhibited cholesterol absorption without altering bile acid turnover.[111] Combined administration of clofibrate and diosgenin to rats produced combined effects on serum lipoproteins.[112] Digitonin treatment inhibited cholesterol absorption in rats and prevented the rise in serum cholesterol in cholesterol-fed cynomolgus monkeys.[113]



10

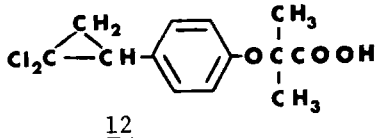


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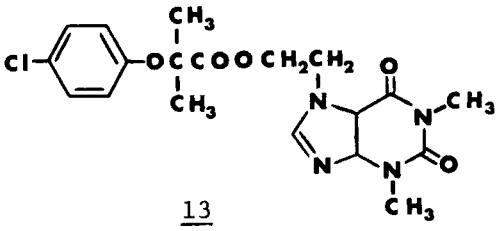
THD-341 - The tricyclic diterpenoid THD-341 (11), at an oral dose of 3 mg/kg or as 0.001% of the diet, lowered serum lipids in cholesterol-cholate fed rats.[114] At a dietary level of 0.01%, THD-341 prevented aortic atheroma formation in cholesterol-fed rabbits and inhibited the progression of established lesions in rabbits fed the cholesterol diet.[115]

Ciprofibrate - At oral doses of 0.6-3 mg/kg/day, ciprofibrate (12) lowered serum cholesterol and triglyceride levels in hyperlipidemic rats.[116] When fed in the diet, ciprofibrate inhibited cholesterol biosynthesis.[116]

ML-1024 - An oxyethyltheophylline ester of CPIB, ML-1024 (13) lowered serum cholesterol and triglyceride levels in rats at similar doses as did clofibrate; serum cholesterol levels were lowered in chronically treated minipigs.[117]



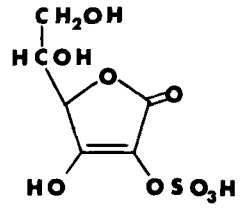
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L-Ascorbate-2-sulfate - L-Ascorbate-2-sulfate (AAS) (14) prevented the increase in serum lipid levels and the accumulation of cholesterol in the aorta of cholesterol-fed rabbits to a greater degree than did ascorbic acid (AA).[118] AAS had similar effects on serum lipid levels in guinea pigs,[119] a species which, like man, does not biosynthesize AA. It was

concluded that the hypolipidemic activity of AAS was independent of the conversion of AAS to AA.[119]



14

Gallstone dissolution - Since the previous discussion of this topic in this series,[120] several reviews have appeared describing the medical treatment of gallstones, especially the use of oral chenodeoxycholic (chenic) acid (CDCA).[121-124] Data on the safety and efficacy of CDCA in over 1000 patients have accumulated in the past 2 years;[125-135] one study comprised 400 subjects.[130] Success rates varied between 50 and 70%, with stones dissolving usually within 4-24 months of treatment. Dose related increases in efficacy[128,130,131,133] and frequency of side effects[127,128] have been shown. The optimal dose is 750 mg/day or 15-17 mg/kg/day;[125,126,128,131,132] obese patients require up to 20 mg/kg/day.[136] Side-effects were minor, the most common being mild diarrhea and elevated transaminases, which tended to normalize after the first month.[130,137] The mode of action of CDCA has been reviewed;[121,122] the result of its activity is to reduce the biliary concentration of cholesterol relative to that of bile acids and phospholipid, thereby reducing the saturation index (SI) of bile.[127] A study on the disposition of the drug[138] showed that CDCA was completely absorbed; the first-pass hepatic clearance of CDCA averaged 62%.

CDCA lowered serum triglycerides in hypertriglyceridemic gallstone patients,[125] and has been used to normalize serum lipids in hypertriglyceridemia, independent of its action on gallstones.[138-141] CDCA decreased triglyceride biosynthesis in hyperlipidemia.[139-141] CDCA enhanced the antihyperlipidemic effect of clofibrate and reduced the clofibrate-induced increase in biliary cholesterol concentration and SI of bile.[132]

Another agent being investigated for gallstone dissolution is ursodeoxycholic acid (UDCA), the naturally occuring 7β-epimer of CDCA.[142-144] No diarrhea or elevated serum transaminases were found at doses up to 15 mg/kg/day.[142-144] The SI of bile was lowered by smaller doses of UDCA than of CDCA.[143,145] Unlike CDCA, UDCA did not elevate lithocholic acid levels in the bile of rhesus monkeys.[146] Although enhanced biliary levels of unconjugated lithocholic acid were not found in humans given CDCA, UDCA may theoretically be safer than CDCA.[147]

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Chapter 20. Somatostatin

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Introduction. The peptide hormone somatostatin has undergone extensive biological and chemical study since its discovery in hypothalamic extracts in 1973.[1] The availability of synthetic hormone[2] has resulted in the recognition of a broad range of biological functions. The synthesis and biological properties of numerous analogs have also been described. Reviews have appeared[3,13,24,72,81] and the proceedings of a symposium on somatostatin has been published.[4]

Biological Findings. Somatostatin was first isolated from sheep hypothalamus and derives its name from its ability to inhibit the release of growth hormone from the pituitary.[1] The availability of the synthetic peptide has led to the recognition that it expresses inhibitory control over a wide variety of endocrine and non-endocrine organs. The use of a variety of techniques (radioimmunoassay, bioassay, immunocytochemistry) has shown that somatostatin is present in other areas of the brain, spinal cord, the D cells of the endocrine pancreas, stomach, the gastrointestinal tract, cells of the thyroid gland, and in the general circulation.[5-10] The presence of somatostatin in these different areas has led some investigators to suggest that the peptide may function both as a hormone and as a neurotransmitter.[11] The original finding that somatostatin, when infused in baboons, caused lowering of blood glucose and concomittant decreases in insulin and glucagon levels,[12] has led to its use in studies attempting to define the role of glucagon in normal carbohydrate homeostasis and in diabetes mellitus.

Somatostatin has been found to inhibit the secretion of various other hormones such as thyrotropin, gastrin inhibitory peptide (GIP), vasoactive intestinal peptide (VIP), pancreozymin, secretin, motilin, gastrin, renin, ACTH in patients with ACTH-secreting pituitary adenoma and ACTH-secretion *in vitro* and to effect exocrine pancreatic function and various GI functions.[3-21] The physiological significance of these findings is not clear at the present time.

From these observations, hypotheses for the development of therapeutic agents based on the actions of somatostatin have emerged. Therapeutic agents could be obtained by the development of analogs of somatostatin having selective inhibitory properties in 1) the endocrine pancreas, 2) the pituitary, and 3) the gastrointestinal tract. The short duration of action of somatostatin, however, is a potential limitation to practical therapeutic applications and will require the development of analogs of longer duration of action or oral activity.[3]

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Effects on the Endocrine Pancreas. The most novel, although speculative, potential application of somatostatin relates to its possible use in the treatment of diabetes and its sequelae.[22-24] Somatostatin has been used as a valuable tool in the investigation of the relative role of glucagon in carbohydrate homeostasis and in diabetes mellitus. During short-term infusion of somatostatin, hypoglycemia occurs accompanied by a fall in glucagon and insulin levels; cessation of the infusion results in a rebound of all parameters. The fall in plasma glucose levels has been shown to be due to a decrease in hepatic glucose production associated with a suppression of glucagon secretion.[25-28] This effect most likely is not by a direct effect on liver, muscle, or fat tissue.[29,30] A report to the contrary has appeared showing *in vitro* effects of somatostatin on glucagon-stimulated glycogenolysis and gluconeogenesis on isolated liver cells.[31] The observed difference may be due to the high concentration of somatostatin used in the latter studies.

According to the bihormonal hypothesis of diabetes mellitus as put forth by Unger,[32-34] the severity of the hyperglycemia has been attributed not only to the relative or absolute insulin deficiency but also to the hyperglucagonemia which is seen in all forms of spontaneous diabetes in man and chemically induced diabetes in animals. Due to insulin deficiency, glucose utilization is impaired, increased lipolysis may occur, amino acids are released from muscle tissue, and the increased production of glucose and ketones is mediated by glucagon. Withdrawal of insulin from juvenile diabetics and subsequent infusion of somatostatin has been shown to result in the prevention of hypergluconemia, in prevention of severe hyperglycemia, and in a rise in β-OH butyrate.[35,36] It has been shown, however, that somatostatin most probably cannot reverse existing diabetic ketoacidosis.[37] Thus, a glucagon suppressing agent such as somatostatin might prove useful in the treatment of diabetes. Somatostatin has until recently been the only glucagon suppressant available for clinical studies. In spite of the short half life and diverse actions, it has been used in a number of interesting clinical experiments which have attempted to ascertain its utility as an adjunct to the therapy of diabetes. Somatostatin in combination with insulin (at doses which did prevent postprandial hyperglycemia or hyperglucagonemia) completely prevented plasma glucose levels from rising after a meal.[38] Gerich *et al*[39] assessed the effect of somatostatin given along with insulin in insulin requiring diabetics. The patterns of postprandial hyperglycemia and the blood glucose variations throughout the day were better during treatment with lowered insulin doses given along with somatostatin than when only insulin was given. A marked deterioration in the serum glucose pattern was observed when somatostatin infusion was withdrawn. Meissner *et al*[40] reported reduced insulin requirements (38-79%) in juvenile diabetic patients when somatostatin was infused via an artificial pancreas along with insulin. A lowering of postprandial blood glucose as well as fewer fluctuations occurred

during treatment. Depressions of GH levels as well as decreased circulating glucagon were observed in all patients. In alloxan diabetic dogs, Dobbs et al[41] have demonstrated improved control of both hyperglycemia and triglyceridemia when somatostatin analogs were administered along with insulin therapy. Wahren and Felig suggested that the blood glucose lowering effect of somatostatin may be largely due to reduced glucose absorption rather than glucagon suppression.[42] Thus, interpretation of the data solely on the basis of glucagon suppression has been questioned.

On the other hand, Unger et al have cited instances where somatostatin reduces the hyperglycemia of diabetes most likely by glucagon suppression alone.[43,44] In these experiments, attempts have been made to eliminate glucose absorption as a variable. Somatostatin lowers fasting plasma glucose levels in insulin-deprived diabetic dogs.[45] Somatostatin also prevents the rise in glucose levels due to glucagon when alanine is administered parenterally.[45] In clinical studies where carbohydrate has been all but eliminated as a factor in the diet, it has been shown that suppression of glucagon along with insulin injections is more effective than insulin alone in the maintenance of euglycemia.[44]

The fact that somatostatin effects nutrient absorption may play an important role in future ideas concerning therapy. The treatment of diabetes with insulin may be thought of as a process whereby one increases the rate of removal of nutrients from the circulation. A new mode of therapy with somatostatin as a prototype may equalize the rates of nutrient entry and removal from the circulation as well as suppress glucagon mediated hyperglycemia. A role for endogenous pancreatic somatostatin as a regulator of nutrient influx from the GI tract in coordination with insulin mediated nutrient disposal has been proposed.[46] Changes in endogenous somatostatin availability per se may be responsible for some metabolic abnormalities.

Although much emphasis has been placed on the development of a somatostatin analog with selected biological activities, an analog which shows insulin, glucagon and growth hormone release inhibiting properties could be used in the postadolescent insulin dependent diabetic. Suppression of GH release may offer utility for the treatment of diabetes because of possible effects of growth hormone on blood glucose[47,48] and the possible role of GH in the development of retinopathies. The desirable properties of a biologically specific somatostatin analog and possible clinical applications have been discussed in detail.[49]

Effects on the Pituitary. Somatostatin inhibits GH and thyrotropin releasing hormone (TRH)-induced thyroid stimulating hormone (TSH) release from the pituitary. No effects on other pituitary hormones were observed in normal subjects.[50] The inhibition of GH release by somatostatin suggests a use in the treatment of acromegaly. The value of GH release inhibition as long-term therpay cannot be predicted.

A further possible application of GH release inhibition by somatostatin is in the control of diabetic retinopathy. Although the etiology of diabetic retinopathy remains undetermined, an attractive working hypothesis is that growth hormone may play a causal role.[47,51-56] Evidence against the GH hypothesis has been cited.[57]

Effects on the Gastrointestinal Tract. Somatostatin inhibits both the gastric secretion induced by gastrin and the release of gastrin,[20] which suggests a potential utility for the treatment of ulcers. Some cases of life threatening GI bleeding have been successfully treated by I.V. administration of somatostatin.[58] A comparative clinical trial of somatostatin with cimetidine in the control of bleeding is in progress.[59] Another important possible application is in the treatment of pancreatitis since somatostatin inhibits exocrine pancreatic secretion.[60] Recent discussions of potential gastrointestinal applications of somatostatin have appeared.[4]

Biological Evaluation of Somatostatin Analogs. In order to evaluate synthetic somatostatin analogs biological model systems for the above effects are required. The most widely studied properties of somatostatin analogs relate to the lowering of insulin, glucagon, growth hormone and gastric secretion. No single system has been used routinely for the study of all parameters. Growth hormone release inhibition is most commonly measured in an *in vitro* primary pituitary cell culture system.[1] This assay is closest to measuring receptor interaction since metabolism is minimized and transport problems do not exist. A direct receptor system for somatostatin on a pituitary cell system has become available recently.[61] *In vivo* evaluation of growth hormone release inhibition is usually studied after subcutaneous administration of somatostatin or analogs in animals having GH levels elevated by pentobarbital or arginine administration.[62,63]

An *in vivo* system for simultaneously measuring the inhibition of glucagon and insulin release in rats both with[64] and without[65] arginine stimulation has been described. A further test modification involves simultaneous continuous infusion of analog and arginine for 30 minutes.[66] These differences in experimental design may lead to apparently different conclusions about the same compound.

Somatostatin Analogs.

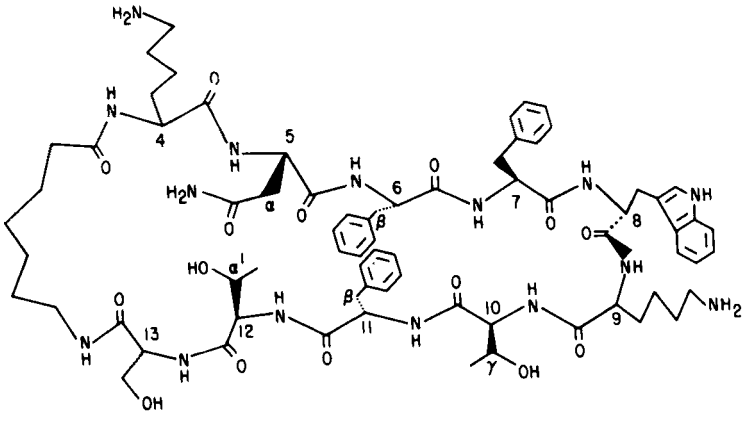
H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH*
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Somatostatin

*All chiral amino acids are of the L-configuration unless otherwise noted. Standard abbreviations for the amino acids have been used throughout. Aha=ω-amino heptanoic acid and Abu=γ-amino butyric acid.

Analogs of somatostatin are being prepared in a number of laboratories with the objective of obtaining compounds of therapeutically useful duration of action and/or improved biological specificity. These two objectives are convergent if a mechanism for improved biological specificity involves attaining selective resistance to metabolism in the area of one type of receptor (such convergence has been observed in the oxytocin-vasopressin series and has been reviewed.)[67]

The first approach undertaken toward increasing the duration of action of somatostatin analogs was through acylated (e.g., acetyl and propionyl) somatostatin derivatives of reduced solubility.[68] These analogs as well as somatostatin given by subcutaneous administration show durations of activity of three hours or more.[69,70] Reduction of the rate of metabolism by amino peptidases through the addition of glycine residues (up to 3) to the amino terminus has resulted in increased duration.[71] Replacement of various amino acids by the D-enantiomer has also been tried in an attempt to reduce metabolism. Varying degrees of success have been obtained by replacement of Gly-2 by D-Ala and Lys-4, Trp-8, Cys-3, Ser-13, and Cys-14 by the corresponding D-amino acids. Other approaches to increased resistance to enzymatic degradation have included deletion of the two amino acids at the amino terminus,[73] deletion of the ensuing amino group of Cys-3,[73] elimination of the terminal carboxyl of Cys-14,[71,74] and replacement of the reduceable disulfide by methylene groups.[74] All of these latter modifications along with substitution of D-Trp in position 8 have been incorporated in analog 1.[70,75] However, the reason for observed increased duration is not always clear.[70] For example, the increased activity of D-Trp somatostatin is observed *in vitro* as well as *in vivo*, pointing to factors other than metabolic stability being responsible for the enhancement of activity.[76a]



1

Another approach to increased metabolic stability can be through the introduction of conformational constraint. Increased understanding of the importance of conformation in peptide bioactivity has occurred during recent years.[76b] Polypeptides in general are likely to exist as a rapidly equilibrating mixture of a number of conformations. If the conformation or conformations which interact with biological receptors happen to differ from those which interact with rate limiting metabolizing enzymes (generally peptidases), then the introduction of conformational constraint could result in increased duration of action. It is also possible that the receptors for different biological parameters will interact differently with various conformations. Thus, in addition to the more obvious possible introduction of biological selectivity through changes in chemical (receptor binding) features, this goal might also be achieved through conformational constraint. Because the possibility exists that even the smallest changes in a side chain may result in a change in the relative stabilities of various conformations, it is necessary to discuss structure-activity relationships for somatostatin while also considering possible change in conformation. Structure modifications in a peptide can be placed in three categories: 1) those certain to impose a conformational consequence (a new covalent bond is introduced), 2) those likely to introduce a conformational consequence, and 3) those unlikely to introduce a conformational change. Most modifications placed in category 3 are so placed on the basis of bias rather than proof, since evidence of the conformation at the biological receptor regarding the whole molecule would be required in order to make a positive assignment. No such evidence exists for any peptide analogs.

The change of chirality on replacement of an L-amino acid by its enantiomer might be expected to change side chain relationships. Interchange of chirality with retention of biological activity must, therefore, be considered a source of possible information about the conformation of the natural hormone at the biological receptor. The high potency of analogs of somatostatin having D-tryptophan in position 8[77] can be understood in terms of a type I β-turn, involving residues 7, 8, 9, and 10.[78] In this conformation, the side chains of residues 8 and 9 will bear an equatorial relationship to the peptide backbone. A change at position 8 to the D configuration could result in a change in the backbone conformation to a type II' β-turn,[79] thereby retaining the equatorial relationship of the side chains of residues 8 and 9. NMR evidence for the existence of such an equatorial relationship of the lysine and tryptophan side chains in D-tryptophan containing analogs has been presented, based on an upfield shift of the lysine γ-methylene of about 1 ppm and a correlation of the shift with the conformation at biological receptors.[78] Since the upfield shift is not observed in somatostatin, it has also been proposed that the conformation(s) which interact with receptors represent only a minor contribution to the overall population of conformations in the natural hormone. The change from Type I to Type II' requires a 180° flip of the amide bond suggesting that it is not directly involved in receptor interaction. The upfield

shift of the γ-methylene of lysine in D-Trp 8 analogs can supply evidence regarding the effect of other modifications on the conformation of that part of the molecule, an aid to drawing structure-activity conclusions.

Biological selectivity results when D-Cys replaces L-Cys in position 3[71] (inhibition of insulin release) and position 14[80] (inhibition of glucagon release). No evidence exists relating to effects on conformation or direct effect on receptor interaction for these modifications. Also, the biological selectivity introduced by replacement of L-Lys-4 by D-Lys[71] (selective inhibition of growth hormone) and increased potency on replacement of Gly-2 by D-Ala[71] have not been defined as involving conformational change or other factors. Although it has been proposed that gastric secretion inhibition varies in a parallel fashion with growth hormone release inhibition,[89] this claim is not supported by other studies.[76,90]

Replacement of an amino acid by proline introduces conformational constraint by elimination of rotation about the carbon-nitrogen bond in the 5-membered ring. Proline in positions 5 and 13 is acceptable in the receptor bound conformation while in position 10 it is not.[65,78] Using this information, a conformation capable of receptor interaction has been proposed (1)[65] which is different from conformations proposed based on physical studies in solution.[82] This model led to the design of analogs of the compound 1 having Asn-5 and Thr-12 and Phe-6 and Phe-11 replaced by cystine (2 & 3, respectively).[65] Each of these analogs shows activity at least as great as that observed for somatostatin. The monocyclic precursor to 2 is also highly active, confirming the unimportance of the side chains at positions 5 and 12, as first observed by replacement of each of these by alanine.

Replacement of either Phe-6 or Phe-11 by Ala in somatostatin results in a substantial loss of activity.[81] That high activity is seen when a disulfide bond replaces the two aromatic rings (3), while simple removal of these rings lowers activity, was interpreted by hydrophobic bond stabilization of the active conformation of somatostatin.[65] The high activity of analogs of reduced ring size such as compound 4[76] is understood through compound 2, since the ring of 4 is present in 2. Both 3 and 4 have been reported to show increased duration of action; 4[76] after subcutaneous administration and 3 after intravenous administration. The increased duration of 3 after intravenous administration likely reflects increased resistance to metabolism but could also involve other factors such as reduced excretion rate. 4 and the analog having D-Cys in the carboxy-terminal position are reported to be relatively selective for insulin and glucagon release inhibition, respectively.[76] The latter is being studied clinically.[83] Other possibly more selective analogs of reduced ring size were also reported by the Salk group.[76] The combined conclusion from analogs 2, 3, and 4 appears to be that most of the potency of somatostatin results from the side chains of residues 7-10, although some contribution to overall potency may reside outside this region.

Cyclo (Aha-Lys-Cys-Phe-Phe-D-Trp-Lys-Thr-Phe-Cys-Ser) 2

Cyclo (Aha-Lys-Abu-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-Ser) 3

H-Cys-Phe-D-Trp-Lys-Thr-Phe-Cys-OH 4

Cyclo (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys) 5

Cyclo (γ-Abu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Glu) 6

A bicyclic analog involving acylation of the amino terminal Ala by the carboxyl of Cys-14 (5) has been reported to be selective for inhibition of growth hormone release.[62] Other growth hormone selective analogs have been claimed through replacement of the disulfide bridge by an amide linkage (6).[84] Another change in the nature of the ring of somatostatin through deletion of Asn-5 has shown selective lowering of insulin and growth hormone.[85] Relative glucagon and growth hormone selectivity was also claimed on replacement of Lys-4 and Asn-5 by His residues.[86] These claims are not based on analysis of relative potencies or ED_{50}'s, nor are they supported by statistical analyses. However, selectivity appears to exist at some doses. An analog, (7), which is almost totally selective for inhibition of insulin release, has been reported.[76]

H-Ala-Gly-Cys-Lys-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-D-Ser-Cys-OH 7

The most critical amino acids appear to be Trp-8 and Lys-9, based on various structure activity studies.[71] A variety of substitutions in the indole ring of position 8 are acceptable and even give analogs of increased potency.[87] In all cases the D-analog is more potent than the corresponding L-analog. The nature of the heterocycle is important, however, since replacement by 3-(1 or 2 napthyl)-L-alanine results in a loss of activity.[88]

Conclusion. Possible therapeutic applications of somatostatin analogs exist for the treatment of diabetes through suppression of glucagon release to attain improved control of blood glucose levels. Inhibition of growth hormone release may be of value in the prevention of diabetic retinopathy and in the treatment of acromegaly. Inhibition of gastric secretion may be of value in the treatment of ulcers and gastrointestinal bleeding. The studies of analogs of somatostatin have shown substantial progress toward increased duration of action and improved biological specificity. Analogs of value for human experimentation have been discovered. The progress of the biological and chemical studies sustains the hope of further advances toward therapeutically useful analogs.

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Chapter 21. Neutral Proteinases in Rheumatoid Arthritis

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Introduction - The most widely held theory of the pathogenesis of RA is that in response to as yet unknown antigenic stimuli, lymphoid cells in the synovium form follicles and begin to produce immunoglobulins such as IgG and IgM (rheumatoid factor). Rheumatoid factor combines with IgG to form insoluble immune complexes which are found both in the synovial membrane and synovial fluid. These immune complexes are thought to initiate the inflammatory responses commonly associated with rheumatoid arthritis. Immune complex activation of the complement cascade leads to the production of permeability and chemotactic factors. These molecules in turn induce the active migration of large numbers of polymorphonuclear leukocytes (PMN) into the affected joints. As the disease continues, mononuclear cells including T and B lymphocytes, plasma cells, and macrophages become the predominant cell types in the joint space. In addition the synovial lining becomes markedly hypertrophied. Thickening is compounded by a layer of granulation tissue, composed of fibroblasts, blood vessels and inflammatory cells, called pannus. Pannus may eventually cover the surface of the cartilage, with significant destruction occurring underneath the leading edge of advancing pannus. As the disease progresses, cartilage and bone and tendon undergo significant degenerative changes associated with severe chronic RA.[1,2] The destruction of the joint connective tissue elements, proteoglycans and collagen, has been shown to be mediated through the action of enzymes at neutral pH derived from the invading inflammatory cells (particularly PMN[3-16] and macrophages[17-21]), proliferating pannus,[2,22-26] cartilage,[27-29] and bone.[30-34]

Proteoglycans in cartilage exist as aggregates of large molecular weight (MW) polyanionic species of 100 or more glycosaminoglycan chains (primarily chondroitin 6-sulfate) attached O-xylosidically to the serine residue of a large protein core.[35] Degradation of proteoglycans at neutral pH usually involves proteolytic activity along the protein core of the molecule.[4] This cleavage leads to proteoglycan solubilization releasing glycosaminoglycan chains, which facilitates the susceptibility of collagen to degradation. Collagen is the major protein component of cartilage and bone. In these structures it exists principally as an insoluble aggregate with a relatively long half-life. The degradation of collagen fibers can be initiated by proteases which degrade the non-helical peptide regions of collagen thereby eliminating certain intermolecular cross links. Degradation of the remainder of the collagen molecule requires the activity of a collagenase which acts at a site specific for the enzyme. Once this cleavage occurs, the molecule denatures and becomes susceptible to non-specific proteolytic digestion.[36]

PMN - The PMN is a phagocytic cell which contains enzymes that are stored in two types of cytoplasmic granules. Azurophil granules contain lysosomal hydrolases, neutral proteinases and bactericidal elements.[5] Specific granules contain collagenase, lysozyme and lactoferrin. PMN release the contents of their granules during phagocytosis. Release may also occur when the cells are trapped within the joint space and undergo spontaneous autolysis.[3,4] Three PMN neutral proteinases have thus far been isolated and characterized; elastase, cathepsin G, and collagenase.

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Elastase - PMN elastase is a serine protease that is active against azocasein, chloroacetyl-3-naphthoic acid-O-toluidide (ClAc-O-NapAS-D), elastin-orcein, N-carbobenzoxy-alanyl-2-naphthol ester, and N-α-tert-butyloxycarbonyl-L-alanine paranitrophenyl ester.[5,9,10] It has been demonstrated that elastase causes complete release of proteoglycan matrix from intact articular cartilage and can degrade isolated aggregates or subunits of proteoglycan in solution.[4] PMN elastase also increases cellular migration to the site of inflammation by liberating complement derived chemotactic products.[12] It can also cleave intermolecular cross links of collagen.[4] Affinity chromatography has been used to purify PMN elastase and it has been demonstrated that the enzyme is present as three isoenzymes with MW's of 33,000-36,000.[10]

Cathepsin G - Leukocytic cathepsin G is a chymotryptic-like serine protease that has been demonstrated to break down proteoglycans and type II collagen from articular cartilage at neutral pH.[4] Cathepsin G, unlike elastase, was not shown to have any effect on soluble type I collagen.[4] The properties demonstrated for cathepsin G which perpetuate the inflammatory response associated with RA include: (1) cleavage of the third and fifth components of complement, generating chemotactic products; (2) direct stimulation of cell migration and phagocytosis at low concentrations; and (3) inhibition of cell migration and phagocytosis at high concentrations.[12] Cathepsin G is highly charged and low MW (av. 25,500). Purification, using affinity chromatography on Sepharose-Trasylol has shown cathepsin G to exist as three isoenzymes.[13] Cathepsin G is a nonspecific protease with activity against substrates such as azocoll, denatured casein and ClAc-O-NapAS-D. It can be distinguished from elastase by its activity on N-benzoyl-D,L-phenylalanyl-2-naphthyl ester.[13-15]

Collagenase - The PMN collagenase is a thiol-metalloproteinase that specifically degrades native collagen fibrils at neutral pH.[36] Collagenase is stored within the specific granules of the PMN in both active and latent forms.[5] Purification of PMN collagenase involves Sephadex G-75 and BioRex-70 chromatography and preparative electrophoresis in polyacrylamide gel. Two collagenases thus purified have MW's of 42,000 and 33,000. It has been suggested that each of the enzymes contains two subunits. The amino acid composition of the two collagenases is almost identical.[9] Latent PMN collagenase has not yet been purified although partial purification of collagenase from synovial lining cells indicates this molecule is probably an enzyme inhibitor complex. Activation of latent PMN collagenase can occur with nonspecific proteolysis or proteolytic activity derived from RA synovial fluid.[16]

MACROPHAGE - Macrophages are also phagocytic cells which enter the joint space in response to chemotactic stimuli. Upon activation they release a variety of mediators into the local environment. Macrophages can be activated by inflammatory stimuli such as endotoxins, lymphokines from antigen- or -mitogen-stimulated lymphocytes, phagocytosis of non-degradable particles such as zymosan or latex and intraperitoneal induction by such exogenous stimulants as thioglycollate and carrageenen. Important products secreted as a result of activation include pyrogens; prostaglandins; complement components C2, C3, C4, and factor B; and proteolytic enzymes active at neutral pH such as plasminogen activator, collagenase, and elastase.[17-20]

Plasminogen Activator - Plasminogen activator is particularly important in perpetuating RA because plasmin formed from plasminogen can

activate complement which results in products that are chemotactic, opsonic, and macrophage activators.[17] Plasmin is also important in collagenolysis by serving as an activator of latent collagenase.[17] Mouse peritoneal macrophage plasminogen activators have been found to be serine proteases, sensitive to inhibition by diisopropyl fluorophosphate (DFP) and the specific trypsin active site blocker p-nitrophenyl guanidino benzoate (NPGB). Two species of plasminogen activator with MW's of 48,000 and 28,000 have been identified.[18]

Collagenase - Collagenase released from activated macrophages is a typical metallo-enzyme mostly present in a latent form which can be activated by other proteases such as trypsin and plasmin.[2,19] Activated collagenase is inhibited by chelating agents (EDTA, O-phenanthroline, penicillamine), disulfide reducing agents (cysteine, dithiothreitol), and serum α_2-macroglobulin. DFP, soybean trypsin inhibitor (SBTI), leupeptin, 4-chloromercuribenzoate, and NPGB are inactive.

Elastase and Nonspecific Neutral Proteinases - Mouse macrophage elastase is also a serine protease responsive to DFP and phenylmethylsulfonylfluoride. Mouse macrophage elastase has a narrower range of activity than the elastases from pancreas and neutrophils. For example, it does not attack synthetic substrates such as $Ac(ala)_3$-4-nitroanilide, or ClAc-ONapAS-D. It can however degrade elastin and azocasein.[20] A neutral protease derived from rabbit bone-marrow macrophages in culture has been isolated. It is metal dependent ($Co2^+$, $Zn2^+$) and inhibited by EDTA, 1,10-phenanthroline, cysteine, and serum, but not DFP or 4-hydroxymercuribenzoate. Its apparent MW is 17,000.[21]

SYNOVIAL TISSUE ENZYMES - The interface between articular cartilage and proliferating pannus represents the area in which maximal progressive destruction of the joint occurs in RA patients.[22] Rheumatoid synovial tissue produces large quantities of collagenase. The collagenase released by synovial cells is predominantly in an inactive or latent form.[36] The inactive collagenase is probably an enzyme-inhibitor complex and not a true zymogen.[2,24,25,36] Active rheumatoid synovial collagenase is a metalloprotease with molecular weight of 33,000. It is inhibited by chelating agents, thiol reagents and the serum inhibitors α_2-macroglobulin and β_1-anticollagenase.[25] A monospecific antibody to human collagenase has localized the enzyme extracellularly around cytoplasmic extensions of dendritic cells and intracellularly within a few macrophage-like and fibroblast-like cells.[25]

Plasminogen activator similar to the macrophage enzyme appears to be present in cultures of RA synovium.[2]

Rabbit synovial fibroblasts in culture have been shown to secrete a metal dependent neutral proteinase that degrades the protein core of cartilage proteoglycans. The enzyme is found in a latent form in the culture medium and is activated by limited proteolysis.[23]

CARTILAGE AND BONE ENZYMES - Neutral proteases, including a collagenase, are released by cartilage cells (chondrocytes) in the presence of products of lipopolysaccharide stimulated macrophages. They are inhibited by dithiothreitol and EDTA, but not by soybean trypsin inhibitor.[27] Four forms of a metal-dependent neutral proteoglycanase from human articular cartilage have been isolated. They have MW's of 26-28,000 (dimers MW 13-14,000) and require zinc or cobalt for activity, and are therefore inhibited by O-phenanthroline and EDTA.[28] Morphological changes in cartilage from RA

patients show that proteoglycan is diminished around most of the chondrocytes, and digested collagen fibrils appear intracellularly.[29] These changes seem to corroborate the biochemical evidence of dissolution of proteoglycan and collagen by chondrocyte enzymes.

Bone resorption occurs when osteoclasts are activated to produce proteases and collagenase by parathyroid hormone or by osteoclast activating factor (derived from phytohemagglutinin stimulated lymphocytes). Although trasylol and soybean trypsin inhibitor do not affect bone resorption, a specific cartilage-derived anti-collagenase seems to be active.[30,31]

Two neutrally active metalloproteases, distinct from collagenase, have been found in bone.[32] They are in a latent form and can be activated by either 4-aminophenylmercuric acetate or trypsin. One is specific for gelatin and azocoll, but not collagen, proteoglycans, or azocasein. The other degrades proteoglycans, azocasein, and azocoll, but neither collagen nor gelatin.[32]

Mouse and chick bone explants have been shown to produce a latent collagenase.[33,34] In addition, the mouse bone explants produce a latent neutral proteinase. Limited proteolysis of these molecules gives rise to proteoglycan and collagen degrading action. Latency may be due to the presence of an enzyme inhibitor complex. The chick latent collagenase has an apparent MW of 54,000, while the active form has an MW of 43,000.[33] Trypsin activation of both latent mouse enzymes decreases their MW's from 60-70,000 to about 40,000. The mouse neutral protease is inhibited by EDTA, cysteine, and serum.[34]

INHIBITORS - The search for new agents which modify the rheumatic process has recently been oriented toward finding inhibitors of the enzymes directly involved in joint destruction. These include inhibitors of the serine (elastase, cathepsin G, plasminogen activator) and metallo (collagenase, non-collagenolytic) proteinases. These compounds have been derived from both natural and synthetic sources.

Serum - Many of the naturally occurring serum proteins that inhibit the cell derived neutral proteinases have been purified and well characterized.[37-40] Both α_1-anti-trypsin (290 mg%, MW 55,000) and α_2-macroglobulin (260 mg%, MW 725,000) can inhibit plasmin, collagenase, elastase, and cathepsin G, although there are differing opinions on the efficacy of α_1-anti-trypsin against collagenase.[36-39,41-46] Collagenase is inhibited by a specific β_1-anti-collagenase (MW 40,000).[38,47] Another recently described inhibitor of collagenase is platelet factor 4.[48] Cathepsin G is also specifically inhibited by α_2-anti-chymotrypsin (48.7 mg%, MW 69,000).[42] There also exists a 65-70,000 MW plasmin inhibitor in the α_2-fraction of serum.[48-52] Plasmin inhibition also occurs in the presence of anti-thrombin-heparin complexes.[53]

Plant - Inhibitors derived from plant sources include the black bean or soybean inhibitors (Kunitz and Bowman-Birk types),[54-57] lima bean inhibitor,[58] and chickpea inhibitor.[59] These are all unusual double-headed polypeptides, with two independent reactive sites, capable of interacting with both trypsin and chymotrypsin.[60-63] They also are inhibitors of granulocyte elastase and cathepsin G.[14,60]

Microbial - Some of the most interesting protease inhibitors have been derived from bacterial sources such as actinomycetes.[64-66] Of particular importance to inhibition of neutral proteases and collagenases are elastatinal, chymostatin, leupeptins, and phosphoramidon.

Since elastase cleaves the carboxyl side of alanine, the alaninyl group at the terminal carbon of elastatinal [N-(1-carboxy-isopentyl)carbamoyl-α-(2-iminohexahydro-4-pyrimidyl)glycylglutaminyl-alaninal] makes it a specific inhibitor of elastase.[64-66]

Chymostatin, N-[((S)-1-carboxy-2-phenylethyl)carbamoyl]-α-[2-imino hexahydro-4-(S)-pyrimidyl]-L-glycyl-L-leucyl-phenylalaninal, has a phenylalaninyl group at the terminal carbon, which makes it specific for chymotrypsin and cathepsin G, which cleave the carboxyl side of aromatic amino acids.[64-66]

The leupeptins are inhibitors of plasmin, a trypsin-like enzyme. Leupeptins contain argininyl residues at their terminal carbon, and inhibit enzymes which cleave at the carboxyl side of basic amino acids such as arginine or lysine.[14,50] The structure of the most active leupeptin mixture is propionyl-L-Leu-L-Leu-Argininal and acetyl-L-Leu-L-Leu-Argininal in a 3:1 ratio.[64-66]

The N-phosphoropeptide moiety of phosphoramidon (N-[(α-L-rhamnopyranosyloxyhydroxy-phosphinyl)-L-leucyl-L-tryptophan]) specifically inhibits those metalloproteases that cleave the amino side of hydrophobic amino acids. Collagenase cleaves at a specific gly-leu or gly-ileu bond in collagen, and is weakly inhibited by phosphoramidon.[64-66]

A new trypsin inhibitor has been isolated from Cephalosporium sp.[67] A strain of Streptomyces produces elasnin (MW 392) which specifically inhibits human granulocyte elastase (50% inhibition at 1.3μg/ml) but is ineffective against pancreatic elastase, trypsin, chymotrypsin, thermolysin and papain.[68]

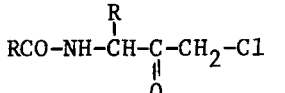
Animal Tissue Inhibitors - Inhibitors of neutral proteases are widely distributed in animal tissues. Low molecular weight inhibitors of leukocyte neutral proteases have been isolated from human tracheal and submaxillary sinus mucosa,[69] and human mucus secretions.[60,70] They are double-headed, with two independent reactive sites, similar to those derived from beans. Cartilage itself has been shown to contain distinct components that inhibit human leukocyte elastase, cathepsin G, and collagenase. The material derived from bovine nasal cartilage is low MW (7,000-serine protease inhibition, and 22,000-collagenase inhibition).[71] Other crude cartilage derived factors inhibit collagenase, regulate osteoclast activity and significantly suppress bone resorption.[30,31,72] Another low molecular weight fraction of bovine nasal cartilage has been demonstrated to inhibit synovial cell surface neutral protease activity necessary for proliferation.[73] Vascular tissue, including aortic extracts[74] and smooth muscle cells in culture[75] are sources of inhibitors of fibroblast collagenase. The aortic tissue material is less than 10,000 MW. While the cultured smooth muscle extract has not been completely characterized it has been shown to affect only mammalian and not bacterial collagenase, is stable to heat and acid treatment, and is sensitive to reduction followed by alkylation.[75] Other inhibitors of fibroblast collagenase have been demonstrated in human skin fibroblast cultures,[76] human tendon in culture[77] and gingival fibroblasts.[78] Collagenase inhibition also occurs with peptide fragments of proteolytically digested tendon procollagen.[79] These results suggest that conversion of procollagen to collagen produces peptides which protect the collagen molecule from digestion by collagenase.

Basic pancreatic trypsin inhibitor (trasylol) has been used in affinity chromatography to purify human leukocyte elastase, however, conflic-

ting reports have appeared concerning its inhibitory effects on human PMN elastase.[80] Trypsin, chymotrypsin and elastase appear to be bound at the same site of the inhibitor.[80] A review of basic protein trypsin inhibitor effects on plasmin and plasma kallikrein has also appeared.[81]

Synthetic Inhibitors - Serine proteases such as elastase and cathepsin G have a charge relay system at their catalytic site involving aspartic acid, histidine, and serine. Specificity among the enzymes arises when secondary amino acids at the active site differ in position, size, or degree of hydrophobicity. The design of inhibitors of these enzymes is based on the enzyme's specific requirements for substrate.[82] Irreversible inhibition occurs when covalent bonds form to the histidine and/or serine moieties of the enzyme, facilitated by the removal of good leaving groups such as Cl^-, F^-, and p-nitrophenol. Specific inhibitors, such as peptide chloromethyl ketones,[82] aza-peptide p-nitrophenyl esters,[82] sulfonyl fluorides (aryl and peptide)[82-84] and lactyl derivatives of 2-aza-alanine peptides[85] have been synthesized in the hope of finding specific serine protease inhibitors. Thiol protease inhibitors are being investigated as inhibitors of collagenase,[86-90] and substituted benzamidines may be of use in inhibiting trypsin-like enzymes, such as plasmin.[91-93]

a) Chloromethyl Ketones - Several chloromethyl ketones (1) have been demonstrated to be specific inhibitors of leukocyte elastase and/or cathepsin G. The fifth amino acid subsite is of primary importance and the enzyme interacts well with succinyl or methyl succinyl groups. The best granulocyte elastase chloromethylketone inhibitor is MeO-Suc-Ala-Ala-Pro-ValCH_2Cl.[82]



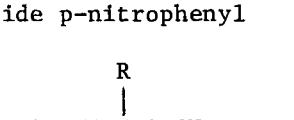
(1)

R = peptide or amino acid

Granulocyte cathepsin G is not inhibited by Tos-PheCH_2Cl, but Z-Phe CH_2Cl and Z-Gly-Gly-PheCH_2Cl are fair inhibitors, with the best inhibitor being Z-Gly-Leu-PheCH_2Cl.[82] The carbobenzyloxy group can be replaced by an acetyl group, but Ac-Ala-Ala-Pro-ValCH_2Cl is much better than MeO-Suc-Ala-Ala-Pro-ValCH_2Cl against cathepsin G.[82]

Chloromethyl ketones also react with thiol proteases, and could therefore be useful collagenase inhibitors.[82]

b) Aza-peptide p-Nitrophenyl Esters - Aza-peptide p-nitrophenyl esters (2) where the α-CH is replaced by a nitrogen atom, are inhibitors of several serine proteases, possibly due to acylation of the active site serine.[82] The most active compound Ac-Ala-Ala-Anle-ONp (Anle = $-NHN(CH_2CH_2CH_2CH_3)CO-$), inhibits both granulocyte elastase and cathepsin G. Ac-Ala-Aphe-ONp acylates cathepsin G, but not elastase.[82]

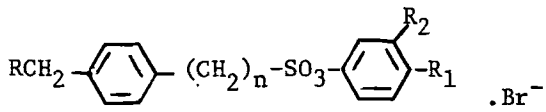


(2)

c) Sulfonyl Fluorides - Serine residues in elastase and cathepsin G are sulfonylated with compounds such as phenylmethanesulfonyl fluoride.[82] Human granulocyte elastase reacts faster with sulfonyl fluorides, while porcine pancreatic elastase reacts faster with chloromethyl ketones indicating that the two enzymes differ in specificities. Human cathepsin G, on the other hand, reacts more slowly than bovine α-chymotrypsin A with sulfonyl fluorides.[82,83]

Attempts have been made to increase the specificity of sulfonyl compounds with respect to degree of inhibition of trypsin, plasmin, thrombin, kallikrein and urokinase.[84] Nitrophenyl esters of benzenesulfonic acid and

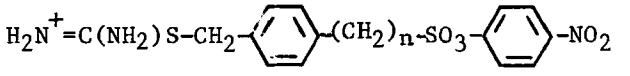
phenylmethanesulfonic acid (3) containing various positively charged groups have been synthesized:



(R = positively charged groups; R^1, R^2 = H or NO_2; n = 0 or 1)

3

Positively charged groups include dimethylsulfide, thiourea, and its N-methyl derivatives, trimethylamine, and pyridine. The best trypsin inhibitor is p-nitrophenyl p'-amidinothiomethylphenylethanesulfonic acid (4),

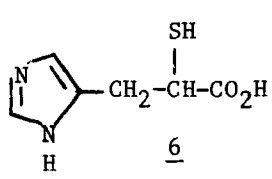


4 n=2
5 n=1

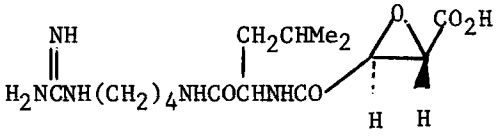
which shows little reactivity toward plasma kallikrein, plasmin, thrombin, with intermediate inhibitory activity directed toward urokinase. The shorter compound p-nitrophenyl-p-amidinothiomethyl benzenesulfonate (5), does not inhibit trypsin, but does inhibit thrombin and to a lesser degree plasmin and urokinase. From these results it is suggested that varying the positively charged groups on these nitrophenyl esters of sulfonic acids can lead to specificity in those inhibitors directed toward various trypsin-like serine proteases.[84]

d) Lactyl Derivatives of 2-Aza-alanine Peptides - Specific granulocyte elastase inhibitors have been found by varying substituents on lactyl derivatives of such compounds as Ac-Ala-Ala-Pro-aza-Ala-Lactyl-X. The lactyl group can be changed to phenyllactyl, but not alanine. The best inhibitors are those where X is $-NHNH_2$ or $-NH_2$, but they do not appear to be hydrolyzed by the enzyme, so acylation of the enzyme probably does not occur.[85]

e) Thiol Protease Inhibitors - Cysteine is an effective inhibitor of collagenase and it has been used in eye drops to prevent collagenase-induced collagen degradation in the cornea.[86] Such compounds as mercaptoimidazolypropionic acid (6)[87] and N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine [from Aspergillus japonicus (7)][88-90] are thiol protease inhibitors.

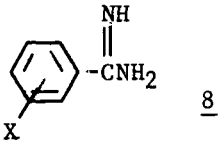


6



7

Substituted Benzamidines - Plasmin and other trypsin-like esterases require specific inhibitors, as exemplified by the broad specificity of some of the sulfonic acid compounds. Substituted benzamidines provide potent and specific plasmin inhibitors.[91] Compounds such as (8), inhibit plasmin and complement C1-esterase.



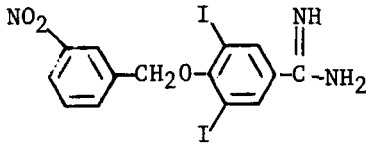
X = alkoxy, H, Br, CH_2OH, CO_2H, NH_2, NO_2

8

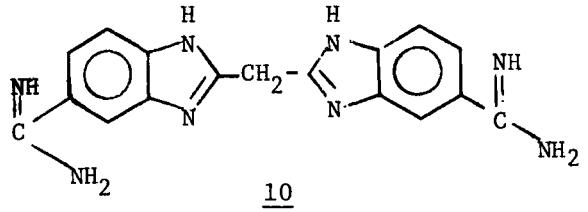
Varying degrees of inhibition are noted depending on electron donation from the substituent and its hydrophobicity.[91]

Substituted (i.e. C:(NH)NH_2, halogen, NO_2, etc.) benzylphenyl ethers, α,ω-dioxyalkanes and α,α',α"-tris(phenoxy) mesitylene benzamidines have been synthesized and analyzed for their inhibition of boar acrosin, a trypsin-like protease in spermatozoa.[92] All of the compounds are more potent than benzamidine, with the most potent compound being 9. While these compounds have not been tested against specific enzymes derived from inflammatory cells, the spectrum of activities of these agents should be ascertained.

Diarylamidine derivatives have been investigated as antiproteases; in 60 out of 62 compounds, one or both of the amidino-substituted aryl moieties included indene, benzimidazole, benzofuran, benzo[b] thiophene and several other related nitrogen-containing heterocycles.[93] One outstanding inhibitor of trypsin (Ki 1.7×10^{-8}M) is bis(5-amidino-2-benzimidazole)methane (10). These compounds demonstrate that variants of small-



9



10

molecular-weight inhibitors can result in specific changes in efficacy with respect to inhibition of protease activity.[93]

CONCLUSION - The development of inhibitors of proteases must take into account such problems as enzyme and tissue specificity, and toxicity (particularly the inhibitor's ability to alkylate or acylate other important body constituents); although in vivo, toxicity may be diminished by homeostatic resynthesis of enzymes and other protein constituents.[94]

In a model of acute inflammatory disease, the reverse passive Arthus reaction, protease inhibitors such as trasylol and SBTI have been shown to diminish the inflammatory response, particularly accumulation of leukocytes and vascular permeability.[95] This provides support for the rational to search for specific neutral proteinase inhibitors as new modalities of therapy in R.A. See also chapter 23.

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Section V - Topics in Biology

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Chapter 22. Proteases and Cell Invasion

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Introduction - Neoplastic cells display a number of biochemical changes compared to their normal counterparts. These may include altered growth controls and requirements,[1] altered rates of glycolysis,[2,3] changes in the composition of the plasma membrane, including protein, lipid and glycolipid modifications,[4,5] increased protease secretion,[6-11] cytoskeletal changes,[12,13] increased cell motility,[14,15] etc. The primary question in cancer biology is what relationship these changes have to those alterations in cell behavior which define neoplasticity. The two features which characterize malignant cells are their invasive and metastatic potentials. While certain normal cells may resemble neoplastic cells with respect to glycolytic rates, motility, and growth controls, only malignant cells are invasive and metastatic. Even cells from benign tumors, which have altered growth controls as evidenced by their tumorigenicity, can be distinguished from malignant cells by their inability to invade and metastasize.

The biochemical alterations which provide malignant cells with the ability to invade adjacent tissues and to spread to other locations are poorly understood and are undoubtedly complex. This complexity manifests itself clearly in the process of metastasis, in which cells must go through a series of steps including tissue invasion, extravasation into blood vessels, thrombus formation, arrest, penetration through the wall of a blood vessel and growth, all in order to develop a secondary tumor.[16] Each step of this sequence may require the precise temporal expression of specific biochemical functions, and some of these reactions, necessary for a step such as invasion, may be detrimental to other steps, such as the formation of a thrombus and arrest.

The process of invasion may be less complex than that of metastasis, since invasion is only one part of the series of steps which comprise metastasis. In fact, cells can display this aspect of the malignant phenotype and not others. Thus, while all metastatic tumors are invasive, not all invasive tumors are metastatic: basal cell carcinomas, for example, are highly invasive but never metastasize.

A number of mechanisms have been suggested to explain the process of tumor cell invasion in terms of observed properties of neoplastic cells. One of these, that tumor invasion is the result of cellular multiplication and mechanical expansion, visualizes tumor infiltration as resembling the growth of a plant root. According to this theory, the pressure from a constantly increasing cell mass is thought to cause

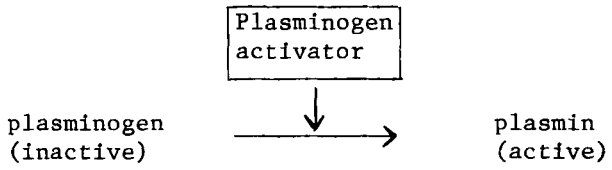
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fractures in the tissue. These fractures would then permit tumor expansion and invasion.[17] This theory is unsatisfactory, however, since there is poor correlation between tumor growth rate and invasive potential. Moreover, certain normal cells such as leukocytes, invade without cell division.

A second hypothesis to explain invasion proposes that the invasive potential of tumor cells derives from their increased mobility and/or decreased adhesiveness.[18,19] These two alterations would change the contact relationships of cells, permitting cells to invade surrounding tissues. However, since the increased motility of tumor cells appears to be random, not directional, their increased motility, per se, would not lead to invasion. In addition, many normal cells which are not invasive are quite motile.

The destruction of surrounding tissues and/or extracellular matrix by lytic enzymes produced by tumor cells has also been proposed to account for the invasive potential of tumor cells. Hydrolysis of the microenvironment by these cells would destroy the natural, physical barriers to cell movement, and would presumably allow migration of cells into neighboring sites. Ultrastructural evidence from in vivo studies strongly supports this view.[20] For example, in the case of epithelial tumors, which are separated from normal tissue by a basal lamina, tumor cell infiltration is always observed to occur in areas of lysis of and damage to the basal lamina. Presumably, the breakdown of the basal lamina, as well as stromal lysis, are mediated by lytic enzymes. The numerous reports of the production of proteases by tumor cells are consistent with such a scheme.[8,9,21-31]

Plasminogen Activator and the Transformed Phenotype: In Vitro Studies - One protease which increases in many cell types after neoplastic transformation is plasminogen activator (PA). The synthesis of PA may be elevated 50-300 fold after transformation. PA is an active serine protease which cleaves at arginine bonds. The enzyme can be inhibited by di-isopropylfluorophosphate, nitrophenyl-guanidobenzoate, and benzamidine.[32] PA efficiently converts the zymogen, plasminogen, to the active protease, plasmin.



No other natural substrates have been found for PA. Plasminogen is a component of plasma and also occurs in extravascular spaces.[33] Moreover, since plasmin is a protease with broad substrate specificity and since the amount of circulating plaminogen is relatively high (50 µg/ml), the production of PA may result in extremely high local levels of effective proteolytic activity, due to the amplification achieved by the local conversion of plasminogen to plasmin.

The evidence that increased protease production, especially PA

production, is related to the malignant phenotype derives from two sources: from in vitro experiments with transformed cells and from experiments with non-malignant cells or tissues which take part in remodeling and/or invasive processes. A large number of experiments in the first category have indicated that properties associated with the transformed cell phenotype in vitro, such as the ability to grow without anchorage,[34,35] increased migration from the edge of a wound[15], decreased cytoskeletal organization,[36] increased agglutinability[37] and morphological changes,[9,37] may be mediated by PA and/or plasmin production. In general, the experimental approach has been to show that under conditions of heightened proteolytic activity, the transformed phenotype is accentuated, while under conditions of decreased proteolytic activity, the transformed phenotype is suppressed.

Although the bulk of the in vitro experiments show a strong correlation between PA production and the transformed phenotype, there are exceptions.[30,38-43] Certain normal cells produce PA, while some transformed cells do not; some cells, which synthesize high levels of PA, grow poorly without anchorage, while some cells which grow well without anchorage, appear to produce low levels of PA. Although these examples of exceptional cells cannot be ignored, certain aspects of the experiments which yielded these results are open to criticism. A lack of accurate quantitation, the use of cell populations with variable karyotypes, and the failure to measure cell-associated proteolytic activity could explain some of the discrepancies. In addition, the failure to assess the possible role of protease inhibitors produced by the same cells, and the use of culture media with inappropriate hormonal compositions are possible explanations of results obtained.

Non-Malignant Systems for the Study of Cell Invasion - The significance of protease activity during the invasive events of metastasis is further supported by the results from studies of the role of proteases in certain non-malignant biological processes. In vivo and in vitro analyses of inflammatory reactions and normal invasive or remodeling events and their hormonal and/or temporal controls have underlined the correlation between protease secretion and the local breakdown of tissue organization. In all such instances, the event and also the associated proteolysis and tissue breakdown have been found to be coordinately controlled. We devote the remainder of this review to a discussion of some of these studies, since they have not been summarized elsewhere.

1. Ovulation - The release of the ovum during ovulation in the rat is almost certainly the result of proteolytic events in the preovulatory follicle.[44,45] PA has been shown to be produced by granulosa cells in response to exposure in vivo or in vitro to physiological levels of the gonadotropins that induce ovulation. PA production is also stimulated in these cells by the in vitro administration of agents which mimic the effects of gonadotropins by increasing intracellular cAMP levels. In vivo, PA is elevated only in granulosa cells from preovulatory follicles, and not in cells from non-preovulatory follicles. The following sequence of events during ovulation in the rat can be postulated based on the precise temporal correlations between gonadotropin stimulation, PA secretion and ovulation. Plasminogen, present in follicular fluid at a concentration equal to that in serum,[46] is activated to plasmin just

prior to ovulation by the gonadotropin-induced generation of PA in selected follicles. This plasmin dislodges the ovum from its attachments to the follicle wall and loosens the granulosa cell layer, clearing it completely away from the area of future follicle wall rupture. The basement membrane, connective tissue, and thecal layers of the follicle wall which are thus exposed are then digested by plasmin, perhaps together with collagenase, ultimately allowing the contents of the follicle, including the ovum, to ooze out.

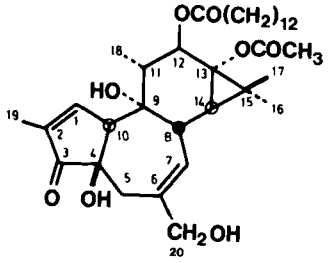
2. Implantation - During a limited period, from days 6-10 of gestation in the mouse embryo,the trophoblast cell is the invasive cell par excellence; it brings about extensive, though localized, destruction of the connective tissue of the uterine wall and invades this tissue to establish an implantation site for the embryo. While the hormonal and/or other controls of this precisely timed event are not understood, Strickland et al.[47] have made an in vitro study of proteolytic events associated with preimplantation mouse blastocysts. They found that trophoblast cells produced both cell-associated and secreted PA, and that this activity was detectable only in the four-day period during which the mouse trophoblast is invasive in vivo: it appeared on equivalent gestation day 6, peaked at day 8, and fell off by day 10. The time-course of PA appearance was unrelated to the emergence of the embryo from the zona pellucida. Microdissection and immunodissection were used to separate trophoblast cells from cells of the inner cell mass in vitro, and the two cell types were cultured separately. The PA was found to be associated only with the invasive trophoblast cells.

The authors suggested that the tissue-destructive activity of the trophoblast is probably plasmin-mediated, though the source of the plasminogen remains unclear; relative to PA alone, the plasmin produced by plasminogen activation would provide a tremendous amplification of both the enzymatic activity and the substrate range, in keeping with the extensive tissue destruction observed during implantation.

3. Inflammation - Macrophages arise from precursors in the bone marrow and circulate in the blood as monocytes, but become activated and migratory in response to infection, inflammatory stimuli, and foreign body carcinogens. These stimuli induce young cells to migrate actively to the site of stimulus. To respond properly, these cells must be able to emerge from blood vessels and carve paths to the appropriate tissue spaces, a process which has several features in common with what must occur during tumor cell invasion and metastasis.

Several recent studies of macrophage responses to inflammatory stimuli and suppressants, in vivo and in vitro, have linked the effects of these agents to their ability to modulate the synthesis and secretion of PA by these cells. While normal, unactivated macrophages secrete barely detectable levels of PA, young, newly recruited macrophages from mice with experimentally induced inflammations produce and secrete as much as 10 times that amount.[48] Furthermore, in vitro exposure of macrophages to conditioned medium from activated lymphoid cells, to Concanavalin A, known to stimulate several functions in these cells. or to phorbol

myristate acetate (PMA), a potent irritant, inflammatory agent, and tumor promotor, further increases PA synthesis and secretion several-fold.[49,50]



PMA

Suppression of PA synthesis and secretion by macrophages *in vitro*, and suppression of the recruitment of these cells, in response to inflammatory stimuli, *in vivo*, can be brought about by physiological levels of anti-inflammatory steroids, such as dexamethasone, hydrocortisone and fludrocortisone. The effectiveness of these steroids in the suppression of PA production is directly related to their *in vivo* anti-inflammatory potential.[51]

These observations point to the physiological importance of PA secretion and subsequent plasmin formation by macrophages in the recruitment phase of the *in vivo* inflammatory response. While other enzymes of these cells, including other proteases, are affected by some of the agents tested, none shows a spectrum of responsiveness to modulating agents that fits as well with the requirements of macrophage migration.

Almost identical findings have been reported for PMN leukocytes, with respect to their involvement in the inflammatory response[52]

4. *Mammary Gland Involution* - During pregnancy, the mammary gland secretory epithelium is elaborated, along with supporting connective tissue, to form ducts and alveoli capable of milk production. At parturition, a period of lactation ensues, and at weaning there is a rapid involution of the mammary gland to its pre-pregnancy state. In mice, as much as 90% of the glandular weight, mostly protein, is removed during one week of involution. Since the rapid and extensive degradative events associated with involution cannot be correlated with any observable changes in lysosomal enzyme activity or in glandular populations of phagocytic cells, at least in the initial stages, the findings of Ossowski *et al.*[53] which correlate epithelial PA production with the remodeling events of involution, are of particular interest.

These authors have found an absolute correlation between the onset of involution and heightened PA production by glandular epithelium, both *in vivo* and *in vitro*. They found a large peak of PA production within 24 h of the cessation of lactation, accompanying the onset of involution, and a gradual decline of glandular PA levels to baseline within the first week of involution. PA production was observed to be temporally coupled to the initiation of involution, even when the onset of involution was moved forward experimentally by the separation of the mother from her litter, or backward, by the administration of oxytocin or corticosteroids, which maintain the gland in its lactating state even

after weaning. Furthermore, in organ cultures of mammary gland fragments, the same coupling of PA production and gland maintenance or involution was observed. Factors or groups of factors which maintained the differentiated characteristics of the tissue in its lactating state, and which prevented involution, such as hydrocortisone, aldosterone, or polyamines in the presence of prolactin and insulin, prevented PA increases over baseline levels; factors which promoted glandular degeneration in these cultures, such as epidermal growth factor or insulin, especially after prolactin potentiation, induced high PA levels.

The specific induction of PA production in secretory epithelial cells in response to signals for mammary gland involution is envisioned by these authors to initiate involution as follows. Secretion of PA leads to increased levels of extracellular PA, which locally activates plasminogen available in the circulation, tissue fluids and milk. Plasmin generated in this manner then degrades, or at least initiates degradation of the basal lamina, the glandular epithelium and any residual milk, and surrounding matrix, restoring the gland to its pre-pregnancy state.

Interestingly, preliminary studies of neoplastic mammary tissue have shown both a several-fold increase in the PA levels of this tissue, as compared with involuting normal glands, and a decreased responsiveness to hydrocortisone.[53] These findings lend further support to the notion that it is the loss of regulation of PA production (and perhaps of proteolysis in more general terms) that is crucial in generating the pathological remodeling characteristic of the malignant phenotype.

5. Rheumatoid Arthritis - Joint tissue destruction in rheumatoid arthritis, a non-malignant biological process involving major tissue degradation and remodeling, involves plasmin-activated collagenase as well as PA-generated plasmin. Rheumatoid arthritis is characterized by synovial cell proliferation followed by extensive cartilage, bone, tendon, and ligament breakdown in affected joints. An invading, proliferating granulomatous pannus advances by proteolytic destruction of tissue in its path.

Dayer et al. have shown that rheumatoid synovial cells in vitro produce elevated levels of collagenase (in a latent form) as compared to the same cells from normal joints.[54] Werb et al. have further shown that rheumatoid synovial cells in vitro produce and secrete high levels of PA.[55] They have constructed in vitro systems to show that collagenase from synovial cells, in its inactive form, binds to collagen types I and II, the types which occur in articulating cartilage and bone regions. Once bound, the latent collagenase is activated by plasmin. In vivo, plasmin is generated from synovial fluid plasminogen, enriched in rheumatoid fluid, by the PA from synovial cells and perhaps also from infiltrating phagocytic cells. Latent collagenase does not bind α_2-macroglobulin. The bound, activated collagenase may also be protected from inhibition by ambient α_2-macroglobulin and other inhibitors both sterically and by competitive binding of the inhibitors by plasmin in solution.

The correlation between the destructive events of rheumatoid arthritis and the generation of PA, plasmin, and collagenase has been

further strengthened by the finding that the production of both collagenase and PA by synovial cells in vitro is reduced markedly by glucocorticoids which act as anti-inflammatory agents in vivo.[55] The effective doses and relative efficacies of these modulating agents are identical in vivo and in vitro. The same relationship between PA production and anti-inflammatory steroids has also been shown for macrophages and PMN leukocytes, as reviewed in section 3.

The collagenase activity seen in rheumatoid arthritis may not be unique to this system. On the contrary, collagen breakdown is important in any major degradative, invasive or remodeling process (also see section 6). The efficiency of plasmin in activating a latent collagenase precursor, and the fact that, where PA is produced, the ready availability of plasminogen almost certainly assures its local activation to plasmin, make this an extremely attractive scheme to invoke in the foregoing examples of non-malignant remodeling events and also in malignancy. The possibility that increases in collagenase activity also occur in these processes is certainly worth exploring.

6. Resistance of Cartilage to Invasion - A particularly interesting example of the potential involvement of proteases in tumor invasion is the documented resistance of cartilage to tumor infiltration. Numerous descriptions exist of tumors which had destroyed a portion of bone but which left adjacent cartilage intact.[17,56] It has been speculated that the resistance of cartilage to invasion derives from the presence of high levels of inhibitors of plasmin, collagenase and thiol proteases found in this tissue.[57-60] Crude preparations of protease inhibitors prepared from bovine or human cartilage are claimed to inhibit a variety of processes, including cell growth, tumor-mediated bone degradation and tumor cell invasion.[61-63]

The avascular nature of cartilage may also be related to the presence of the same molecules which protect cartilage from tumor invasion, for the process of neovascularization has many aspects in common with tumor invasion.[64] Intact cartilage fragments as well as cartilage extracts are capable of inhibiting angiogenesis in vitro and in vivo[65,66]. Moreover, procedures which extract the protease inhibitors from cartilage render the tissue susceptible to both tumor-induced vascularization and tumor invasion.[67]

These observations, as well as the facts listed below, have led us to propose that both neovascularization and tumor invasion require the production of two proteases: plasminogen activator and collagenase. These points are:

1) Neovascularization always occurs from venules.[68] These structures are known to be leaky, and the leakage of a plasma component, e.g., plasminogen, may be required for the invasive process.

2) The first morphological event observed during neovascularization is the destruction of the basement membrane adjacent to endothelial cells, which subsequently migrate.[68] Neighboring endothelial cells then divide to form a new blood vessel. The basement membrane is composed primarily of Type IV collagen and glycoproteins, and the lysis of this structure indicates the production of specific proteases.

3) The major structural protein of cartilage and many other tissues is collagen. An invasive tumor or endothelial cell would have to produce collagenase to degrade this protein, since collagen is resistant to other proteases.

4) Stimulated endothelial cells are capable of synthesizing both PA and procollagenase.[69,70] The PA produced may convert plasminogen to plasmin, and plasmin may activate procollagenase to collagenase.

Thus, we can postulate a mechanism whereby the concerted activities of plasmin and collagenase may degrade the basement membrane glycoproteins and Type IV collagen. Therefore, high local levels of inhibitors of collagenase and plasmin, such as those found in cartilage, might prevent tumor and/or endothelial cell invasion.

Conclusion - The experiments discussed throughout this review give strong support to the hypothesis that proteases and, in particular, plasminogen activator are involved in tissue remodeling and invasion. The examples which we have presented comprise but a small sample of the various phenomena which might involve proteases. Processes such as innervation, involution of glands such as the prostate and the thyroid, growth of the mammary gland before lactation and during embryogenesis, the invasion of the pericostal bud, and bone remodeling may involve PA and plasmin either directly or indirectly.

Given the proteolytic mechanisms employed for tissue lysis, it is not surprising that pathological situations may develop from the abnormal secretion of PA. One disease which may involve the inappropriate production of PA is rheumatoid arthritis. Tumor invasion is a second example. The evidence that PA secretion provides the biochemical mechanism for tissue lysis and subsequent tumor invasion during metastasis, while indirect, is strong. This does not mean that other proteases are not involved, either in concert or singly, in invasion by certain tumors or cells. However, most proteolytic assays other than the PA assay are not sensitive enough to detect the small amounts of enzyme produced. Therefore, the most meaningful work has been concerned with PA production.

Finally, it is worth noting that the nature of the invasive process, as we present it, is such that invasion could be interfered with by the administration of protease inhibitors of appropriate design. Such molecules with the appropriate tissue and enzyme specificities could potentially provide therapies for a number of normal and pathological conditions.

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Chapter 23. Selected New Developments in the Biochemistry of Viruses

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I. Introduction

The goal of molecular viroligists is to understand the structure, expression and control of viral genes. Control is known to be exerted on transcription initiation, messenger RNA (mRNA) processing, and post-translational cleavage of viral coded proteins. Recent information on viral genomes, mRNAs, and the processing of viral products is briefly reviewed here. Where possible, novel implications for diagnosis or viral control are included.

II. Restriction Endonucleases

Restriction enzymes are permitting rapid advances in understanding viral DNA genomes.[1] Also, specific genetic functions can be isolated and located in specific fragments of DNA. And the considerable variation that exists among different strains of viruses is being recognized and utilized for the first time.

A. A Genome Map - Probably the most complete genome map has been determined for the small transforming papovavirus SV40. An excellent review covers this information.[2] The virus has a DNA genome with an estimated molecular weight of 3.4×10^6, and the complete nucleotide sequence of this DNA was recently determined.[3,4] Several proteins have been matched precisely with their coding regions on the DNA and the sequences of several others have been predicted.[3,4] The following points have been established. The site of the origin of replication of the viral DNA has been determined to be near a 27 base-pair palindrome, the center of which serves as the only cleavage site of the restriction enzyme Bg/I.[3] Nearby, transcription begins. Roughly one-half of the genome is transcribed before DNA replication begins to form two (early) proteins. The other half is transcribed after DNA replication begins to form three proteins which are viral structural (late) proteins.[2,3] Replication and transcription occur in both directions from their sites of origin. About 15% to 23% of the genome information is not translated into proteins; possibly it contains regulatory functions.[3,4] The information for the two early proteins (20,503 and > 90,000 daltons) starts at nucleotide 80 from the center of the palidrome.[3] The large early protein is the T antigen. Direct reading of the information for the smaller early protein stops at nucleotide 602. The larger protein shares about one-half of the information of the smaller protein, then obtains the rest of its information from a non-contiguous region of the DNA.[3,4,5] A non-translated region of DNA lies in between and the mRNA for the larger protein is processed by splicing.[3,4,5] The genetic information for two of the three late viral proteins overlaps. Messenger RNA molecules for two of them also are formed by splicing. The different messenger RNA molecules apparently contain a common 5' leader sequence.[3]

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B. Locating Gene Functions - The goal of mapping a mutant is to determine what function is affected by the mutation, and where that function is located on the physical map. Consequently, the protein or protein region responsible for the function can be determined. The quest to identify the gene function(s) required for viral transformation and how that function is controlled has been of special interest.

1. Temperature-sensitive Mutants - A number of temperature-sensitive mutants of SV40 have been analyzed.[2] Those found defective in the complementation A group and the physical map regions which code for the early proteins (T antigen and t) were also found defective in their ability to transform cells.[2] The temperature-sensitive transformation defect was reversible at permissive temperatures, i.e., transformed colonies appeared. Mutants defective in the formation of late proteins (viral structural proteins) were able to transform cells. Therefore, functional early proteins are required to effect and maintain transformation.[2] The early proteins necessary for transformation also permit viral DNA replication and the stimulation of host cell DNA synthesis. The exact biochemical functions required for transformation have not yet been determined.

2. Deletion Mutants - Another approach has been the construction of deletion mutants by the judicious use of restriction enzymes and appropriate exonucleases.[2,6] The sites of the deletions were mapped; in some instances they were widely separated but still affected a single protein.[5,6] The functional ability of the mutants was then determined. The results, while at an early stage, are in agreement with the conclusions reached with the temperature-sensitive mutants. The early proteins must be functional to effect DNA replication, stimulation of host cell DNA synthesis and cell transformation.[5] Interestingly, not all three of the late proteins appear to be required for virus production.[2] Much more will surely come from these studies.

3. DNA Fragments - Fragments of DNA can be taken up by cells under proper conditions and in many cases still express their genetic functions. A fragment of DNA consisting of 74% of the SV40 genome and containing those parts of the genome coding for the early proteins and the origin of replication was capable of transforming cells.[7] The minimal amount of information required for transformation and the nature of the product of that information is expected from future studies of this type.

This approach has been used with the adenoviruses, also. In summary, DNA fragments representing the left terminal 4.5% of the adenovirus genome can transform cells.[8] The transformation was somewhat abnormal but normal transformation was achieved with the left 7.5% fragment.[8,9] Viral messenger RNA molecules were isolated from cells transformed by fragments. They were translated *in vitro*. Four proteins between 33 and 40 thousand daltons resulted from the translated RNA from the cells transformed with a 4.5% fragment. These four and an additional protein of 19 thousand daltons resulted from the mRNAs from the normally transformed cells. It is puzzling that such large proteins resulted as a large number of stop codons were found when the 4.5% DNA fragment was sequenced.[8] The large number of proteins found in this system indicates its complexity; considerably more work is required to understand the roles of the proteins in cell transformation.

C. Finding and Analyzing Integrated Viral Genes - Restriction enzyme digests of the DNA from several independent SV40 transformants were analyzed to see whether the SV40 genome was integrated similarly and at one or more sites on the cellular DNA. Not only did integration occur at more than one cellular site but different sites of the viral DNA were joined to the cellular DNA in each of the transformants.[2]

Similarly, restriction enzyme digests of cellular DNA have great sensitivity and therefore detected for the first time the presence of mouse mammary tumor virus (MMTV) DNA in infected mammary glands.[11] This permitted the diagnosis of infection of these glands by horizontally transmitted strains of MMTV, i.e., they could be distinguished from those endogeneous to the infected mice. This was possible because the different strains of MMTV differ in their DNA sequences and thus in the fragments produced by selected enzymes.[11] This illustrates the sensitivity and selectivity provided by this technique to identify specific DNA sequences.

D. Comparing Viral Genomes - To exploit the diagnostic potential of restriction enzyme mapping, the degree of variation among the strains of naturally occurring viruses must be determined. Comparisons have begun. Comparison of the restriction fragments of 10 strains of SV40 virus revealed each strain to be unique.[2] The DNAs from rabbitpox, vaccinia, cowpox, ectromelia, and fowlpox all gave distinguishable patterns of fragments when appropriately digested and compared.[12] When Hind III fragments of DNA from various poxviruses were compared, cowpox, vaccinia, monkeypox, and variola were identified.[13] Additional digestion with Sal I permitted 4 of 5 strains of variola virus to be further differentiated.[13]

In other studies, three strains of Epstein-Barr virus[14] and herpes simplex types 1 and 2[15,17] each gave unique restriction enzyme patterns. The herpes simplex case is expected as the two types of herpesviruses differ antigenically. However, a large number of different isolates of herpes simplex type 1 yielded different patterns of DNA fragments when their DNAs were fragmented with several appropriate restriction enzymes.[15,16] Isolates obtained years apart from the same individual and a single isolate after numerous passages in tissue culture retained their unique, characteristic fragment patterns.[17] Thus, this technique might aid in epidemiological studies. Indeed, the analysis of 14 isolates from infected individuals in a hospital unit showed that there were two separate introductions of herpes type 1 viruses into the unit.[17]

The variations found in the genomes of the different strains of all the viruses examined, suggests that we might expect the structures of their gene products to vary considerably, also. This could account for variations in disease severity and suggests that antiviral chemicals may vary considerably in effectiveness.

III. RNA Virus Genomes: Structures, Transcription, and Translation

Viruses which contain an RNA genome can be classed as positive and negative-stranded viruses depending on whether their genome RNA serves directly as mRNA (positive) or requires RNA transcription to synthesize mRNA molecules (negative). Dramatic discoveries have been made in each of these virus group.

A. Positive-Stranded RNA Viruses - The positive-stranded RNA viruses are best represented by the picornaviruses of which poliovirus is a model virus. Poliovirus contains a single-stranded genome RNA, which has a MW of 2.5×10^6 (7700 nucleotides), and four viral structural proteins.[18] Upon entrance into a cell, the genome RNA is translated into one large polypeptide which is cleaved proteolytically to produce all the viral proteins (see below). The 5' ends of picornavirus genome RNAs are unique as they contain a covalently linked protein.[19] The protein (VPg) has a MW of 6-12,000, and has both a basic and hydrophobic nature.[20,21] It is linked to the 5' terminal sequence pU-U-A-A-A-A-C-A-G through a tyrosine-O-p-U linkage.[20,21]

The functional significance of VPg has not yet been determined but it is believed to be involved in RNA replication. This is because VPg is found only in RNA involved in RNA synthesis or packaged in the virion.[21] Since no VPg is found in viral RNA isolated from infected cell polyribosomes,[21] it is presumedly removed prior to or during translation and is probably not required for translation. An enzyme which is able to specifically cleave the tyrosine-uridine linkage leaving both VPg and the genome RNA intact has been isolated from three uninfected cell lines and partially purified.[22]

The 3' ends of picornavirus genome RNAs contain a stretch of adenosine residues known as poly(A).[24] The genome RNA of picornaviruses differs from most mRNA molecules in that its 3' poly(A) tail is transcribed from a poly(U) template during RNA replication rather than synthesized post transcriptionally.[23] Recent determinations of the nucleotide sequences of the 3' RNA region adjacent to the poly(A) of ten picornaviruses indicate no sequence homology between subgroups and only minor sequence homology among the viruses of a particular subgroup.[24] This becomes important when one realizes that the 3' terminal region serves both as the initiation site for RNA replication and the termination site for translation.

B. Negative-Stranded RNA Viruses - The negative-stranded RNA viruses are larger and more complex than the positive-stranded viruses. They contain an RNA polymerase which transcribes their genome RNAs into functional mRNAs. Vesicular stomatitis virus (VSV) is one such negative-stranded virus. Virions of VSV contain a single strand of RNA with a MW of 4×10^6 and five structural proteins. The proteins account for virtually all the coding capacity of the RNA (Review 25). Since VSV must undergo RNA transcription to synthesize mRNAs before translation can occur, transcription has received the greatest attention.

VSV differs from poliovirus in several respects. One major difference is that the VSV genome serves as a template for both mRNA synthesis and RNA replication, i.e., in one instance five distinct species of mRNA result and in the other a single full-length copy of the genome is formed. The controlling mechanism allowing either transcription or replication is not yet known, although breakthroughs have resulted from recent studies. The first was the use of UV irradiated virus to prime either an RNA polymerase assay[26] or a coupled transcription/translational system.[27] A polar effect on synthesis of mRNA was observed. If the five

mRNA species were synthesized independently as was believed for many years, then the UV irradiation would show a non-polar effect. Instead the results indicate that there was a single initiation point and sequential synthesis of the five mRNA species. The synthesis of each mRNA species is dependent on the prior synthesis of its 3' proximal mRNA.

In addition, RNA synthesis is initiated at the 3' end of the genome RNA with the synthesis of a small 48 nucleotide "leader RNA."[28,29] Determination of the complete nucleotide sequence of the leader RNA demonstrated that unlike any VSV mRNAs it contains 50% AMP and a ppA at its 5' end.[30] Two serotypes of VSV and the paramyxovirus, Newcastle disease virus, synthesize leader RNAs of identical size and similar base composition.[31] The VSV leader RNAs share 80% base sequence homology which indicates nearly identical binding and initiation sites for the virion polymerases.[32] The question remains whether the five mRNA species arise by a processing mechanism after synthesis of the leader RNA or a stop-start mechanism in which the RNA polymerase reinitiates at each junction between mRNAs. Similar sequential synthesis of mRNA has also been observed with the paramyxovirus, Sendai virus.[33]

Viral as well as eucaryotic mRNA structures have been intensely scrutinized (see Review 34). Virtually all mRNA molecules contain a "cap" structure at their 5' termini (Figure 1).

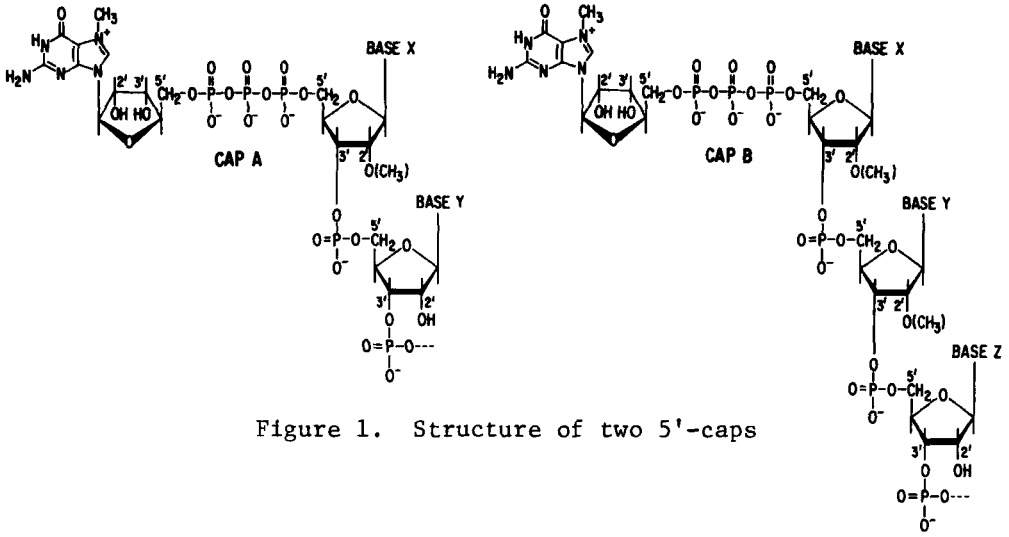


Figure 1. Structure of two 5'-caps

The cap consists of an inverted guanosine residue linked through its 5' ribose to the 5' ribose of the initiated or first transcribed base via a triphosphate bridge as $G_{ppp}X_pY_p...$ The 5'-5' linkage is inverted, relative to the normal 3'-5' phosphodiester bonds in the remainder of the polynucleotide chain. Most viruses can methylate cap structures <u>in vitro</u> in two positions - at the 7-methyl position of the capping base and the 2'-O-ribose position of the penultimate base X to form a Cap A structure (Figure 1). In contrast, the vast majority of viral cap structures synthesized in virus infected cells contain an additional 2'-O-ribose methylation to form a Cap B structure (Figure 1). Caps have been detected in almost all eucaryotic viral mRNAs except for the picornavirus group;

they differ only in the purine of the penultimate base X and presence of 2-O' methylation. The caps on viral RNAs are synthesized in one of two ways - either with a G_{pp} condensing onto a pG as with reo virus[35] and vaccinia virus,[36] or a Gp condensing onto a ppG as with VSV mRNAs.[37] Consistent with their ubiquity in eucaryotic mRNAs, caps have an important role in translation. This was first demonstrated with reo virus and VSV mRNAs which require 5' terminal 7 MeG caps for efficient translation in a wheat germ cell-free, protein-synthesizing system.[38] However, the dependence of eucaryotic mRNA translation on caps appears to be neither absolute nor universal since poliovirus RNA isolated from polyribosomes contains the 5' terminus pU.[21]

A great amount of research has been done recently on the 5' terminal binding sites of viral mRNA molecules (see Review 39). The cap structure, though involved in the binding of mRNA to ribosomes, is not always protected from RNAase digestion during ribosome binding. In fact, despite a large amount of sequencing data, the requirements for ribosome binding and the initiation of protein synthesis are not yet known.

Initiation of translation is restricted to the 5' region, although a maximum allowable distance of the initiating triplet from the 5' end of the RNA has not been defined. It is possible to initiate at an AUG codon located as far as 89 residues in from the 5' end. Although initiation of translation in eucaryotic systems seems to be limited to the 5' portion of the mRNA, it is clear from examination of the available sequence data that the AUG codon need not occur in a fixed position relative to the 5' end and can exist as close as 11 nucleotides in brome mosaic virus mRNA[40] to over 200 nucleotides in SV40 VPI mRNA.[41] Likewise, the sequences which flank either side of the AUG codon do not seem to matter.

IV. Enzymatic Protein Cleavage in Virus Replication

A. Extent of Protein Cleavage in Virus-infected Cells - Probably without exception among the animal viruses, enzymatic cleavage (site-specific limited proteolytic attack) of viral proteins occurs in or on the surface of infected cells. The evidence collected for some of the representative viruses indicates that the cleavages are essential for the successful production of viral progeny (reviewed in 42). At the biochemical level, understanding the nature of these cleavages is leading to a fuller appreciation of the role of limited proteolysis in the activation of enzymes and in the formation of complex supramolecular biological structures. These studies may also lead to the discovery of viral inhibitors which act by inhibiting the required virus protein cleavage reactions.

The occurrence of virus protein cleavages and the possible role of these reactions are summarized in Table I. The most common observation is that limited proteolysis occurs at the level of maturation (formation of infectious virus particles). Mechanistically, the virus structural protein precursor is cleaved, and activated to a form which makes it recognizable to viral nucleic acid and/or cellular membrane receptors. The cleavages occur most commonly within the infected cells, but in several examples final proteolytic "tailoring" occurs after virus has exited the cell, and is in the serum or some other extracellular environment.

With some animal viruses, especially the small, RNA-containing picorna- and togavirus groups, the intracellular cleavages begin on very large "poly protein" precursors, and virtually all virus gene products, including nucleic acid polymerizing enzymes, are regulated proteolytically (reviewed in 43). Thus, these viruses are examples of parasitic agents whose entire replication cycle is dependent on successful activation of several discrete protein gene products by limited proteolysis.

TABLE 1 Characteristics of Virus Group in Which Protein Cleavage Occurs

Virus	Structural Features of Virion	Role of Cleavage
Picorna (Polio)	SS(+)[1]RNA, protein coat.	All viral proteins are cleavage products, most functions regulated by protolysis, Viral protease identified, which processes coat precursor.
Toga (Sindbis)	SS(+) RNA, protein core, glycoprotein-lipid envelope.	Similar to picorna.
Oncorna (Retro) Rous sarcoma	SS(+) RNA protein core, reverse transcriptase, glycoprotein-lipid envelope.	Probably similar to picorna. Evidence for a virus protease, sensitive to -SH protease. inhibitors.
Reo	DS RNA, protein core, outer protein coat, RNA transcriptase.	Activation of transcriptase, maturation.
Myxo (Influenza)	SS(-) RNA, protein core, glycoproteins (hemagglutinin and neuraminidase) and lipid envelope, RNA transcriptase.	Activation of virons. (hemagglutinin)
Paramyxo (Sendai)	Similar to myxo.	Absolute requirement that envelope glycoprotein be cleaved for infectivity.
Rhabdo (Vesicular stomatitis)	SS(-) RNA, "bullet-shape" nucleoprotein core, matrix protein embedded in lipid membrane, outer glycoprotein.	Removal of "signal sequence" on glycoprotein, during transport through membranes.
Adeno	DS DNA, numerous protein structures in capsid.	Maturation. Virus protease?
Pox (Vaccinia)	DS DNA, many proteins and lipid in capsid.	Maturation.
Herpes simplex	DS DNA, protein core, proteins and lipid in envelope.	?
Papova (SV40)	DS DNA, three proteins in capsid, histones in core.	Capsid protein synthesis, T-antigen production, (transformation).
Parvo (Adeno-associated virus)	SS(+) DNA, three proteins.	Capsid protein synthesis and assembly.

[1]Abbreviations: SS-single stranded, DS-double stranded, (+)-infections (messenger RNA-like), (-)-complementary, transcribed into messenger RNA.

B. Origins of the Proteolytic Enzymes

1. Host Cell Proteases Used by the Infecting Virus. It is obvious that for a complete understanding of the processing reactions the origin of the participating protease(s) must be known. Although unambiguous proof is still lacking, there are indications that host cell proteases participate directly. Most fully described is the activation by a cell membrane-associated protease of an envelope glycoprotein precursor of the paramyxoviruses, e.g., Sendai virus (reviewed in 44). Cells, which apparently lacked cleaving activity, produced virus particles with very low infectivity. One of the envelope glycoproteins in these virions was an uncleaved form. Growth of the virus in a high yielding cell gave infectious virions with virtually all the glycoprotein in a cleaved form. Cleavage allows the virus to proceed successfully through the early stages of infection to the point where the viral nucleic acid is released into the host cell. A compelling finding was that trypsin, or other proteases, can replace the missing function of the non-producing cell. Similar results were reported for a paramyxovirus, type 1 isolated from brain tissue of human patients suffering from multiple sclerosis. The virus, produced in brain tissue, was very low in infectivity, unless treated with trypsin or mixed with macrophages. It was suggested that the intrusion of proteolytic macrophages into brain tissue harboring the virus could trigger off a burst of virus activity leading to the symptoms of MS.[45]

2. Virus-coded Proteases. Recent data support the view that a protease is a virus gene product. Examples include the RNA tumor viruses,[46] togaviruses,[47,48] adenoviruses,[49] and picornaviruses.[50] Although it is still problematic whether the protease identified is truly of viral origin (or an activated cell enzyme), the data clearly indicate a large quantitative induction of proteolytic activity upon infection.[50,51] Genetic and cell-free studies[48,52] further support the virus-coded hypothesis. Perhaps the most elegant examples are those in which messenger RNA from picorna- and togaviruses have been translated in cell-free extracts to yield proteases synthesized de novo.[48,52] These studies should be useful in finally assigning the proteolytic activity to a virus gene product.

C. Inhibition of the Proteolytic Reactions - The reactions leading to cleaved viral proteins may be blocked in infected cells by at least two distinct strategies.

1. The substrate, i.e., the virus precursor polypeptide, may be altered so that it cannot be cleaved. This has been accomplished by incorporation of amino acid analogs,[53] incubation at abnormally high temperatures,[54] blocking glycosylation of the precursor glycopeptides[55] (e.g., by deoxyglucose or tunicamycin treatment), and by zinc ions.[56] The last is a particularly simple, useful technique, and is reversible in some examples.[57]

2. Chemical inhibitors of proteases may be used to inactivate the participating enzymes. Iodoacetamide and iodoacetate[52,58,59] prevent processing of virus proteins. Diisopropyl fluorophosphate, probably acting as an inhibitor of a serine active-site protease, also prevents the processing of poliovirus polyprotein.[60] Finally, chloromethyl ketones of selected amino acids, particularly that of phenylalanine, have been widely used in successful tests to block viral protein processing.[60,61] The

viral protease which cleaves picornavirus capsid protein precursors is specific; leucine residues donate the new -COOH group (reviewed in 42). Based on this, a recent study used a leucine chloromethyl ketone to specifically inhibit capsid protein production in poliovirus-infected HeLa cells.[51] It is possible that, as careful characterizations of the virus-specific proteases become available, synthetic efforts will lead to antiviral chemicals which inhibit their function.

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Chapter 24. Liposomes as Drug Carriers

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Introduction - Liposomes are vesicles composed of one or more lipid bilayers completely surrounding an internal aqueous space. They are usually composed of phospholipids either in pure form or in combination with other amphipathic molecules such as sterols, long chain bases or acids, or membrane proteins. The structure of liposomes varies from large (0.5→5μ) multilamellar vesicles[1] to small (∿300 Å) unilamellar vesicles.[2,3] More recently, new methods have been reported describing the formation of unilamellar vesicles of intermediate size.[4,5,6] The general properties of liposomes and their interaction with various macromolecules have been described in several reviews.[7,8,9]

The main features of liposomes that have made them a valuable investigative tool are the following: (1) their characteristic morphology, where a relatively impermeable membrane completely encloses an aqueous space and (2) their ability to encapsulate various solutes present in the aqueous phase during their formation. These characteristics prompted their initial description as a model membrane system[1] which was followed by an ever increasing number of studies important in understanding biological membrane structure and function. It was the same characteristics that also prompted the use of liposomes as carriers of drugs and other macromolecules.[10,11,12]

The concept behind the use of liposomes as carriers of drugs and macromolecules is related to an expected protection of the encapsulated molecules in the blood stream, an altered tissue distribution and pharmacokinetics, as well as an increased uptake into cells by mechanisms that are not normally available for these molecules. Some of these expectations have been verified through studies in various laboratories during the last few years. Such studies have shown that liposome encapsulation can alter drastically the pharmacokinetics and tissue disposition of the encapsulated substances, it can enhance their uptake into cells, and it can increase their pharmacological efficacy. Several recent reviews have discussed these early results in considerable detail.[13-20]

A critical evaluation of the early literature indicates reasons for both optimism and caution in relation to the future use of liposomes as drug carriers. The simplicity of the initial concept can lead to an underestimate of the technical difficulties of liposomes preparation and the biological complexities in relation to the interaction of liposomes with cells in various tissues. It seems clear that the ultimate utility of liposomes as drug carriers will depend largely on further methodological developments and a better understanding of their interactions with cells. Progress in this area will enable us to produce liposomes with the appropriate properties suited to a particular application and, thus, optimize their effectiveness. A conference on liposomes and their uses in biology and medicine was organized recently under auspices of the New York Academy of Sciences.[21] The conference included subjects ranging from physical chemistry of liposomes to their interactions with cells in vitro

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and their use as drug carriers in tumor-bearing animals. This article will summarize briefly the most significant recent studies.

Methodology for Liposome Preparation - An informal agreement was reached on the use of a three-letter acronym to designate the type of liposome such as multilamellar vesicles (MLV) or small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV) with the chemical composition in parenthesis after the acronym (Ref. 21, p. 367). The term liposomes is therefore to be used as a generic name to include all types of artificial vesicles composed of phospholipids and other amphipathic lipids.

The original liposome preparation of Bangham et al[1] consisting of multilamellar vesicles (MLV) has been admirably suited in defining many membrane properties and was the basis for the development of the sonicated unilamellar vesicles (SUV). However, both preparations show a relatively low volume of entrapped aqueous space per mole of lipid and restricted ability to encapsulate large macromolecules. This is because in MLV most of the lipid is participating in the internal lamellae, and the close apposition of the adjacent concentric bilayers restricts the internal water space. In SUV, which are single-compartment vesicles, the ratio of surface area to encapsulated volume is so large that only a small aqueous volume per mole of lipid can be attained. Attempts to circumvent these shortcomings have been only partially successful. The ethanol injection method produces vesicles of about the same size as SUV with the same shortcomings.[22] The ether infusion technique produces large unilamellar vesicles with high captured volumes per mole of lipid,[5] but the efficiency of encapsulation is relatively low. Two recent reports describe technical improvements of the ethanol injection[23] and ether injection methods.[24] Other useful techniques for preparing large volume vesicles either use specialized conditions[25] or are restricted to a single phospholipid.[4] Methods based upon solvent evaporation have been attempted in the past but have resulted in the formation of multilamellar vesicles.[26-28] A method designed to form asymmetric vesicles by centrifugation of a suspension of dense aqueous inverted micelles through an organic solvent/water interface has been reported.[29] This report, however, indicated that the internal volume was small and the vesicles themselves relatively unstable. Techniques based upon the removal of detergents yield vesicles slightly larger than SUV.[30] Recent modifications of the cholate detergent dialysis technique of forming small unilamellar vesicles[31] include removal of the detergent by gel filtration[32] and fast controlled dialysis system.[33] These are suitable for membrane reconstitution experiments but, like the SUV, fail to encapsulate the aqueous phase efficiently. Recently, a method that combines detergent dialysis and solvent evaporation has been described.[34] This technique leaves an equal weight of detergent per phospholipid in the resulting vesicles, making them unsuitable for many biological applications.

We have been concerned by the fact that none of the above methods can produce liposomes with all the following desirable characteristics: (i) the ability to entrap a large percentage of the aqueous material; (ii) a high aqueous space-to-lipid ratio; and (iii) widely variable chemistry of lipid components without detergents. A new method[6] was recently developed which meets to a large extent all the above criteria and is based on evaporation of a water-in-oil emulsion (REV). It is relatively easy

to establish in most laboratories and produces vesicles of intermediate size (0.2-0.4μ), which can encapsulate with high efficiency most water-soluble drugs and large macromolecules.

The most recent methodological improvement involves the production of liposomes of well-defined size distribution by extrusion of MLV or LUV through a nucleopore polycarbonate membrane.[35] The pressure extrusion method allows the sequential passage of preformed liposomes through membranes of decreasing pore diameter. During the extrusion, all the lipid material passes through the membrane, but the size of liposomes is reduced to approximately the diameter of the membrane pores.[35] This procedure can be applied to any type of liposomes, and it is rapid and simple. If the extrusion is performed in the presence of the mother liquid used for the initial encapsulation, it results in relatively good encapsulation efficiency.

Interactions of Liposomes with Cells In Vitro - The first observations on the ability of liposomes to affect cell behavior in vitro include the work of Magee and Miller[36] who found that liposomes carrying anti-viral antibody could protect cells against viral infection, the work of Papahadjopoulos et al,[37] who found that liposomes could induce cell fusion without cytotoxic effects, and the work of Gregoriadis and Buckland[38] who found that liposomes containing invertase could cause the disappearance of vacuoles of stored sucrose in mouse peritoneal macrophages. The initial observations on the ability of liposomes to induce cell fusion generated interest on the question as to whether liposomes could fuse with the cellular plasma membrane.[12,39,40] Such a mechanism of uptake would result in a much more efficient delivery of drugs into the cellular environment, compared to the more conventional endocytotic pathway of pinocytosis or phagocytosis.[14]

The mechanism of interaction of lipid vesicles with cells in culture has been studied in detail during the last few years in several laboratories, and the results were reviewed recently.[18,19,41] The evidence has been obtained with various types of vesicles and cells under varying conditions, and it therefore seems contradictory in several cases. There is general agreement, however, that liposomes are taken up quite efficiently by cells in culture, that the process of uptake is temperature dependent, but in most cases it does not require metabolic energy, and that the vesicle contents are incorporated along with the lipids. In cases where the uptake has been quantitated in terms of SUV liposomes per cell, it appears that several million vesicles can be incorporated within a few hours without overt cytotoxic effects.[12] Concerning the mechanism of uptake, the evidence indicates that it could involve any of the following: fusion with the plasma membrane[12,42-48] endocytosis[43,48,49] adsorption to the cell surface[50,51] or molecular exchange.[42,52] The predominance of any of these mechanisms is controlled by the chemistry and physical properties of the liposomes and perhaps by the cell type and the state of the cell. Since the evidence for most of the mechanism of uptake is indirect and the contribution of each type of uptake is very difficult to quantitate, most conclusions that have been drawn up-to-now should be considered at best only qualitative statements. There is clearly a need for further work in this area, especially in developing quantitative assays for estimating fusion events and distinguishing them from classical endocytosis

or adsorption followed by molecular exchange at the cell surface.

Irrespective of the difficulties in quantitating the contribution from each of the various types of uptake, it is clear that liposomes can be used to enhance drastically the cellular uptake of molecules that are not normally taken up. This aspect of liposome-cell interactions is of obvious importance to their use as drug carriers. The first indication of the dramatic increase of the pharmacological efficacy of an encapsulated drug was the enhancement by 1000 fold of the ability of cyclic AMP to inhibit the growth of 3T3 cells *in vitro*.[12,53] A similar large increase has been observed more recently[54] with actinomycin D against a hamster cell line which exhibited resistance to this drug. Increased cytotoxicity following encapsulation has also been observed with cytosine arabinoside triphosphate.[55] The last two observations raise the important possibility of using liposomes to circumvent resistance to drugs due to decreased cellular transport as well as to metabolic block.

Other important demonstrations of increased cellular uptake *in vitro* following liposomal encapsulation include uptake of horseradish peroxidase,[56,57] hexosaminidase,[49] cytoplasmic localization of carboxyfluorescein,[58] a calcium-chelating agent,[59] secretion in mast cells induced by calcium-containing liposomes,[60] synthetic polynucleotides inducing interferon,[61] messenger RNA inducing globin synthesis,[62,63] polio virus infecting CHO cells[64] and, more recently, isolated viral RNA.[65] The last four studies are important as first demonstrations on the use of liposomes for the incorporation of informational macromolecules into the genetic apparatus of cells.

Although the quantitative aspects of the uptake of liposomes by various cells and the respective mechanism of uptake have not been studied in detail, it does not appear so far that there is a marked cellular specificity. It would, of course, be of obvious advantage for chemotherapy if liposomes could be constructed that have increased affinity for specific cell types such as tumor cells. Several attempts have been made for *in vitro* targeting of liposomes with varying degrees of success. The first such experiments were reported by Gregoriadis and Neerunjun[66] using antibodies raised against cell surface antigens. The presence of the antibody immunoglobulins resulted in an increased uptake which was cell-specific. Similar results were obtained more recently by Magee *et al*[67] who also reported increased cytotoxicity with liposomes containing both antibody and actinomycin D. Increased cellular uptake has also been obtained with heat aggregated IgM by dogfish macrophages which carry the Fc receptors,[57] also with a plant lectin and glycoprotein-containing liposomes by erythrocytes,[68] and finally with DNP-containing liposomes and anti-DNP antibody by TNBS-treated cells.[69] This last study reported that the increased cellular uptake did not result in enhanced cytoplasmic release of carboxyfluorescein from the vesicles. However, their interactions were only short-timed, and the results do not preclude an enhanced cytotoxic effect over a longer period of time as a result of the antibody-specific enhanced cellular uptake. Observations have also been obtained indicating that the presence of specific glycolipids (such as lactocerebroside) enhances the uptake of liposomes by Hela cells in culture.[70]

Tissue Disposition and Pharmacological Effects of Liposomes In Vivo - Since the initial publication of studies involving injection of liposome-entrapped substances in vivo,[11,71,72] there has been an increasing number of studies on both the altered tissue distribution and also on the increased pharmacological efficacy of encapsulated agents. The subject has been reviewed recently.[13,15,20]. The most important points for future consideration include the permeability properties of liposomes in a physiological environment, their interaction with plasma components, the role of liposome size and chemistry in determining the rate of removal from the circulation and their tissue localization at the cellular level.

The permeability of liposomes is an important parameter which depends not only on the innate properties of the lipid bilayer and the encapsulated material,[8] but also on the interaction of liposomes with plasma components[73,74] and various cells.[75] Our own early studies with liposomes indicated that the presence of a high ratio of cholesterol to phospholipid is essential for preventing various proteins from "penetrating" into the bilayer and increasing their permeability.[76] Injection of liposomes composed of phospholipids without cholesterol into mice was found to result in complete release of encapsulated Na^+ and other small molecules.[77] This effect seems to be related to the interaction of liposomes with plasma proteins and lipoproteins.[73,74] The incorporation of a high ratio of cholesterol into liposomes seems to prevent this high degree of leakage, and can also affect drastically the anti-tumor effects of encapsulated ara-C in L-1210 tumor-bearing mice.[78] There was a recent proposal that the permeability properties of liposomes in vitro could also be controlled (enhanced) in specific areas of the body by application of hyperthermia.[79,80] This possiblity is based on earlier observations showing that liposomes composed of saturated phospholipids exhibit a drastic increase in permeability when the temperature is raised to the critical point (Tm) for the melting of the acyl chains.[81] The new technique of gamma-ray perturbed angular correlation seems to be useful for observing the release of liposome contents in vivo.[82]

The effect of liposome size and chemistry on the rate of clearance from the circulation was demonstrated clearly by Juliano and Stamp[83] by the use of well-defined preparations of SUV, which remain in the circulation for much longer periods of time compared to the large MLV. From this and other studies,[84-89] it is clear that blood clearance and tissue distribution of liposomes can be markedly affected by their physicochemical properties. From the work of Kimelberg et al,[88] it appears that encapsulation of methotrexate into SUV not only prolongs its plasma clearance rate but allows for a much broader tissue distribution (compared with MLV; see also Ref. 89) and also reduces drastically its metabolic degradation. MLV are usually cleared rapidly by kidney and spleen, although the incorporation of positive charge has been shown to result in lung accumulation.[86] The interaction of the drug molecules with the lipid bilayer has also been shown to affect the clearance rates and tissue disposition.[90,91] The incorporation of various glycolipids has been shown to result in changes in the tissue disposition of the encapsulated material.[92] Finally, recent studies on the effect of size have shown that very large MLV (>1 micron diameter) are retained preferentially in the lung where they retain their contents for relatively long periods of time.[93] Lung retention was also achieved by infusion of aerosolized liposome preparations.[94] Prolongation

of the clearance rate in mice of encapsulated maltose in SUV has been achieved by the use of dialkyl analogs of phosphatidyl choline.[95] A small increase in the uptake of ^{111}I-labelled bleomycin by tumors in mice has been reported after incorporation of immunoglobulins into liposomes, although the uptake of liver and spleen was also augmented.[96] The uptake of bleomycin-containing liposomes into neoplasms in human patients has been followed in one reported case.[97] Other *in vivo* studies with antibody bearing liposomes have also been reported.[98,99] The role of liposome structure on their (relatively limited) ability to enhance oral absorption of drugs has been discussed recently.[100-103] Localization of the contents of positively charged liposomes in arterial lesions has been reported recently.[104]

The interaction of liposomes with various plasma components is an important parameter not only in relation to their permeability but also in defining the chemistry of the surface groups available for interaction with cells in various tissues. There is a report indicating that liposomes recovered from plasma have altered electrophoretic mobilities, indicating adsorption of plasma proteins.[105] The same study reported that α_2-macroglobulin seems to be specifically recovered with such liposomes. Other studies indicate that lipid material from the liposomes is recovered in the lipoprotein fraction[74,106] and that lipid material can exchange between liposomes and cells[52,107,108] especially in the presence of exchange proteins. It is therefore important to consider the question of lipid-exchange between liposomes and various lipoproteins and cells in studies where liposome localization is determined by the appearance of radio-labelled lipid in various tissues. This point is important since localization of such lipid[102] may reflect exchange with lipoproteins and may not be strictly indicative of the localization of vesicle contents. In considering tissue localization of liposomes and their contents, the most important question is the bioavailability of such markers. Localization of intact liposomes especially in interstitial spaces in various tissues may not necessarily be accompanied by release of the vesicle contents into intracellular spaces where the drug could act. Techniques should therefore be developed for quantitating not only the tissue localization of vesicle contents but their uptake at the cellular level and their availability for pharmacological action.

The pharmacological effects of encapsulated drugs are, of course, the ultimate test of their bio-availability, although the quantitation of the uptake and the mechanism of drug uptake by the recipient cells is difficult to ascertain by such complex biological endpoints. The effects of various pharmacological agents encapsulated in liposomes have been reviewed recently.[17,20] The most recent and exciting application is the dramatic increase of the therapeutic index of antimonial compounds against a model for leishmaniasis disease following liposomal encapsulation.[109-111] In this case, the effectiveness of the liposomes against the parasitic organisms is presumably achieved because of their mutual residence within the reticuloendothelial cells, which represent a natural targeting system. Other recent applications include effects against inflammation with liposomes injected in arthritic joints,[112] anti-tumor effects of encapsulated methotrexate against lymphosarcomas resistant to this drug,[113] anti-tumor effects of encapsulated alkylating agents[114] and nucleotide analogs such

as ara-C[115,116] and alleviation of respiratory distress syndrome, following aerosol infusion of dipalmitoyl phospholipids.[117] The ability of liposomes to act as adjuvants[118] along with other interesting facets relating to immunological properties of liposomes have been reviewed by Tyrell et al.[15] Use of liposomes for alleviating enzyme deficiencies by delivering the missing enzymes was the pharmacological application that initiated the activities of several groups in the area of liposomes as delivery vehicles.[10,119] Ironically, this has proven a most difficult task, judging from the progress so far.[102,103,120] It may be that with the development of methods for the encapsulation and cellular delivery of RNA and DNA[62-65,121,122] liposomes could ultimately prove valuable for correcting not only enzyme deficiencies but other genetic defects.

Conclusion - From the studies reported so far, it is clear that liposomal encapsulation of drugs and various other macromolecules can have the following important pharmacological effects: (1) increase of cellular uptake of impermeant molecules (shown definitely in vitro); (2) decrease of the rate of plasma clearance of molecules with short half-lives; (3) alteration of tissue disposition with variable tissue specificity depending on the properties of the liposomes; (4) inhibition of the metabolic breakdown of molecules; (5) enhancement of the pharmacological efficacy without overt cytotoxic effects, although the problem of toxicity has been studied in detail only in a few cases.[123,124] The studies that have been published so far permit an attitude of cautious optimism, although it may be said that in the past there has been an underemphasis of the possible problems and difficulties.[125] There is no doubt that much more work is needed in the areas of increased cellular and tissue specificity and the possible toxicity of liposome-encapsulated drugs. It is also clear that further methodological developments are needed before the liposome system can be utilized at optimal conditions. Liposomes were developed originally as a model membrane system and not as drug carriers. Most of the pharmacological applications attempted so far have been obtained by using the original preparative methodology, which has been far from optimal. However, the encouraging success that has been achieved so far can be expected to generate increasing interest in further defining the properties of the liposome system and its optimization suitable to each particular application.

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Chapter 25. Non-enzymatic Glycosylation

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Introduction - The modification of proteins via enzymatic glycosylation is a well-described aspect of biochemistry. Indeed, the ubiquity of glycoproteins alone attests to the general importance of enzymatic glycosylation reactions in biology. Recently, however, it has become apparent that proteins also may be glycosylated by nonenzymatic processes, and that these reactions may play major roles in both health and diseases. The prototype molecule for this process must be considered to be hemoglobin A_{Ic} (Hb A_{Ic}), as this was the first protein recognized to be nonenzymatically glycosylated. The chemistry and biology of this hemoglobin have been the subject of intensive investigation, prompted by the fact that patients with diabetes mellitus have elevated concentrations of Hb A_{Ic}. Studies directed at understanding this observation have provided new insight into the pathobiology and clinical management of diabetes. Thus, this review will summarize the current data concerning the nonenzymatic glycosylation of proteins in humans and related species and focus to a large extent on Hb A_{Ic}.

Structure of Hemoglobin A_{Ic}- The hemoglobins of the adult human erythrocyte may be resolved by ion exchange chromatography on the polymethacrylic acid resin BioRex 70 into several distinct components.[1] Hemoglobin A (Hb A) comprises 90-95% of the total, while at least half a dozen quantitatively minor hemoglobins make up the remainder. Hb A_{Ic} is an acidic minor component that comprises 3-5% of the total Hb in normal individuals, and up to 15% in patients with diabetes mellitus.[2]

Hb A and Hb A_{Ic} have identical amino acid sequences.[3] Initial studies[3] determined the unique structural feature of Hb A_{Ic} to be a low molecular weight moiety attached at the NH_2 - terminus of the β chains via sodium borohydride reducible linkage, presumed to be a Schiff base. Mass spectroscopic data was consistent with a hexose as the blocking group,[4] and recently the structure 1-amino-1-deoxyfructose has been proposed.[5] This was based on the observation that acid hydrolysis of Hb A_{Ic} liberates 0.25 moles of hexose consisting of glucose and mannose in a 3:1 ratio per mole of α, β, dimer, and that periodate cleavage of the product obtained by reduction of Hb A_{Ic} with sodium borohydride ($NaBH_4$) liberates primarily 3H-formic acid, rather than 3H-formaldehyde. The unequivocal assignment[6] of the 1-amino-1-deoxyfructose structure was possible by direct isolation of the NH_2-terminus glycodipeptide following reduction of the β chain of Hb A_{Ic} with $NaBH_4$ and comparison with authentic, synthetic compounds using proton magnetic resonance spectroscopy, gas-liquid chromatography and thin layer chromatography.

Biosynthesis of Hb A_{Ic} - Two mechanisms have been proposed to account for the synthesis of Hb A_{Ic}. Both presume the nonenzymatic formation of a Schiff base between a carbohydrate and the NH_2 - terminus of the β chains, followed by an Amadori rearrangement to yield the 1-deoxy-1-amino-2-ketose product (Fig 1).[7] In the first proposal glucose would react directly with hemoglobin. Since red cells are not insulin dependent for glucose uptake, diabetics have elevated glucose concentrations in erythrocytes and this

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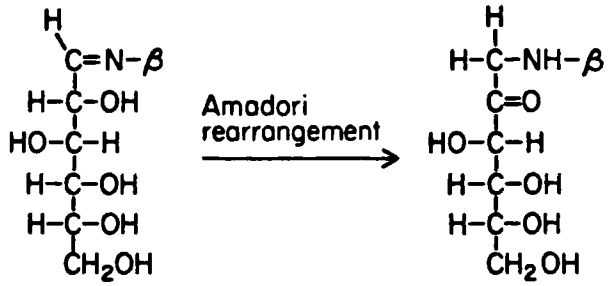


Figure 1

would explain their increased Hb A_{Ic} levels. This hypothesis would however, make it difficult to explain the apparent specificity of the reaction for the NH_2 - terminus of the β chains. The NH_2 - terminal valine of the α chain has a lower pk and hence should be more reactive. Furthermore, lysine ε-amino groups might be predicted to react, albeit at a slower rate. Although the synthesis of Hb A_{Ic} has been achieved by the *in vitro* incubation of Hb A with glucose,[9] other laboratories found this reaction to be slow and not specific for the β chain NH_2-terminus, [10,11] whereas the reaction with glucose-6-phosphate was both rapid and specific. Thus it was proposed that the initial reactant might be glucose-6-phosphate, which would combine specifically due to its affinity for the diphospho-glycerate binding pocket, (DPG) and that a phosphatase would then be required to complete the formation of Hb A_{Ic} [10,11]. In fact, specific adducts between the NH_2-terminus of the β chains and carbohydrates were easily synthesized *in vitro* with any carbohydrate that contained both a phosphate group and an unblocked carbonyl (e.g. glucose-6-phosphate, fructose-6-phosphate, fructose 1,6-diphosphate, glyceraldehyde-3-phosphate; but not glucose-1-phosphate, glucose-1,6-diphosphate, UDP-glucose).[11] The problem with this hypothesis would require diabetic erythrocytes to have elevated glucose-6-phosphate levels, and the evidence on this point is contradictory.[11,13]

A solution to this problem may recently have been found. Whereas the glycosylation of Hb had previously been thought to be specific for the β chain terminus, recent data suggest that Hb A is itself heterogenous and contains molecules with carbohydrate attached at various other amino groups.[14] Thus, glucose may be the reacting molecule and the apparent specificity for the NH_2-terminus of the β chain results from the fact that the adduct at this position happens to have a unique chromatographic mobility, whereas glycosylation of other NH_2-groups does not significantly alter the chromatographic behavior of Hb A.

Investigations of Hb A_{Ic} synthesis *in vivo* have provided a theoretical foundation for the recently demonstrated value of Hb A_{Ic} measurements in clinical medicine. Initial studies,[15] in which wild-type and genetically diabetic mice were injected with ^{59}Fe-labeled reticulocytes, demonstrated Hb A_{Ic} to be synthesized in a slow, nearly irreversible reaction throughout the life of the mature erythrocyte.

Since erythrocytes are not capable of protein synthesis, this has

to occur by the post-synthetic glycosylation of already-formed Hb A. Furthermore, when reticulocytes from wild-type mice were injected into diabetic recipients, the rate of Hb A_{Ic} synthesis increased 2.7 fold (Fig. 2).[15] Human Hb A_{Ic} has also been shown to be synthesized at a slow, constant rate for at least the first 80 days of red cell survival.[16] A steady state concentration of Hb A_{Ic} is apparently achieved via the replacement of old erythrocytes laden with Hb A_{Ic} by new cells relatively poor in Hb A_{Ic}. The actual Hb A_{Ic} concentration is a reflection of red cell half-live and the rate of synthesis, which in turn is a function of the blood glucose concentration. The near-irreversibility of the reaction can be attributed to the stability of the Amadori rearrangement.

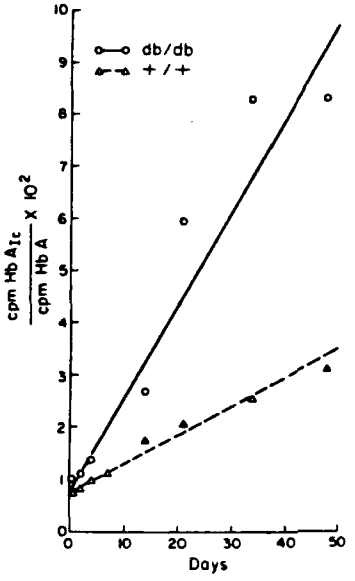


Figure 2
(Reprinted with permission from ref. 15)

Hemoglobin A_{Ic} in the Management of Diabetes Mellitus - A diabetic with severe chronic hyperglycemia will synthesize Hb A_{Ic} at an abnormally rapid rate, and therefore should have a very high blood Hb A_{Ic} concentration. An improvement in diabetic control would immediately decrease the rate of synthesis, but old red cells with high levels of Hb A_{Ic} would remain in the circulation until their 120 day life-span expired. Populations of red cells would be present in the circulation reflecting the mean blood glucose level from the time of the Hb A_{Ic} measurement backwards for several months. In other words, a single Hb A_{Ic} measurement should reflect a person's mean blood glucose level over the previous weeks to months. This hypothesis has now been substantiated by multiple clinical investigations.

In human diabetics, Hb A_{Ic} levels correlate with fasting blood sugars,[17,18] and a high degree of correlation is seen in response to an oral glucose tolerance test.[17] When a series of poorly controlled diabetics was hospitalized for 3-6 months to achieve strict carbohydrate control (mean fasting blood sugar [FBS] decreased from 343 to 84 mg/dl), the decrease in Hb A_{Ic} concentration lagged 3-4 weeks behind the improvement in diabetic control (Fig. 3).[19] To better assess mean blood sugar levels, blood sugar values were measured just prior to and 1 hour following breakfast, lunch and dinner. These values were summed and called "glucose brackets". A highly significant correlation was found between mean blood sugar concentrations, as assessed by glucose brackets, and Hb A_{Ic} values both prior to and during strict control (Fig. 4).[19] Several outpatient studies have demonstrated positive correlations between Hb A_{Ic} and multiple measurements over time of urinary sugar and low post-prandial blood sugar.[20-24]

Since Hb A_{Ic} is a marker for chronic hyperglycemia, its concentration

should be elevated regardless of the cause of the diabetes. Thus, mice with diabetes secondary to specific genetic lesions or administration of the pancreatic β-cell toxins alloxan and streptozotocin all have elevated Hb A_{Ic} concentrations.[25] Hb A_{Ic} levels are independent of the mode of therapy in human diabetes, and in identical twins discordant for diabetes, are elevated in the diabetic siblings.[26]

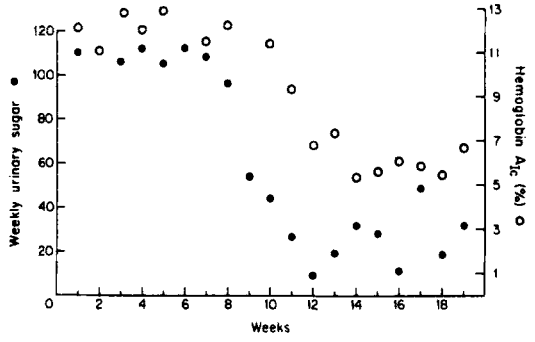


Figure 3
(Reprinted with permission from ref. 19)

Thus, both basic and clinical data show Hb A_{Ic} levels reflect the patient's mean blood glucose level for the preceding weeks to months. Hb A_{Ic} levels are not influenced by short term fluctuations in blood sugar. Infrequent quantification of Hb A_{Ic} concentration in the blood of diabetic outpatients can objectively and accurately assess the quality of carbohydrate control, a feature unique to the measurement. This should allow more optimal adjustment of therapy for diabetics. Furthermore, Hb A_{Ic} measured serially in a prospective study can be used to better define the relationship between carbohydrate control and the development of the sequelae of chronic diabetes.

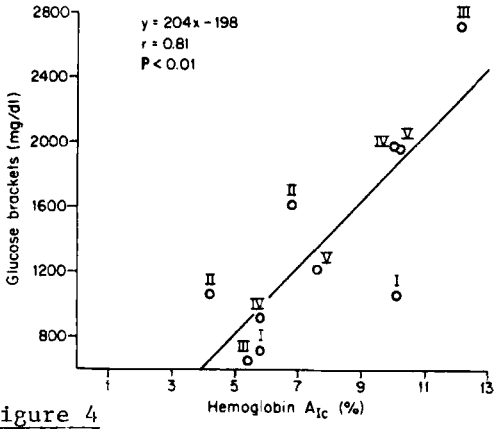


Figure 4
(Reprinted with permission from ref. 19)

Diabetic Sequelae - While the development of insulin replacement therapy has greatly prolonged the diabetic's lifespan, it has not had a major impact in the prevention of the myriad of sequelae associated with long-standing diabetes. Kidney, retina, peripheral nerve, lens and the cardiovascular system all suffer dysfunctions secondary to diabetes. The multisystem nature of this disease has made difficult the development of a unifying hypothesis to explain these sequelae.

The glycosylation of Hb to form Hb A_{Ic} may represent a model reaction to explain the etiology of many of these complications of diabetes. It has been pointed out that these sequelae tend to occur in tissue which do not require insulin for glucose uptake, and therefore that these cells will have intracellular glucose concentrations in proportion to the severity of hyperglycemia.[27] Perhaps proteins, other than hemoglobin are subject to increased non-enzymatic glycosylation in diabetes. Such glycosylation could alter the solubility, enzymatic activity, half-life or other property of the particular protein and thus account for the observed clinical dysfunction.

In support of this is the observation that carbamylation of amino groups by the anti-sickling drug cyanate results in both periphral neuropathy[28] and cataracts,[29] that are histologically similar to those seen in diabetes. It is noteworthy that the cyanate-induced lesions are reversible upon cessation of drug administration,[28,29] perhaps reflecting turnover and implying that the lesions of diabetes would be reversible or at least controllable if euglycemia could be achieved. The rate of reversibility of the sequelae of diabetes would be a function of the glycosylated protein's turnover rate in vivo. In addition, nonenzymatic glycosylation is not completely irreversible and the glyco groups could elute slowly from proteins with very long biological half-lives. In fact, certain sequelae of diabetes, e.g., platelet hyperaggregability, have been shown to be reversible upon institution of rigid carbohydrate control.[30] It should be noted that although Hb A_{Ic} has a slightly elevated oxygen affinity[31,32] this is not likely to contribute to clinical disease since mutant hemoglobins with much greater oxygen affinities are not associated with any of these symptoms.[33]

Nonenzymatic Glycosylation of Other Proteins - Since any free amino group with a sufficiently low pK should have the potential for forming a Schiff base and Amadori rearrangement product, one would predict many proteins in addition to hemoglobin are nonenzymatically glycosylated. Tissues in which diabetic sequelae occur are currently being investigated for the presence and significance of such processes. The proteins of the lens have, to date, been the most thoroughly investigated. Studies of bovine and rat lens crystallins have revealed nonenzymatic glycosylation of lysine ε-amino groups in vitro and in vivo in the presence of a high glucose or glucose-6-phosphate environment.[34] This glycosylation imparts an increased susceptibility of the crystallins to undergo sulfhydryl oxidation to form high molecular weight aggregates, which attain a large enough size to defract light and cause an opacity within the lens. The addition of reducing agents prevents, as well as partly reverses, the formation of these aggregates.

The accumulation of sorbitol within the lens has also been offered as a mechanism for the formation of diabetic cataracts.[35] At the present time it is unclear what the relative roles of these two mechanism are in the genesis of diabetic cataracts.

Recently, the nonenzymatic glycosylation of human albumin both in vitro and in vivo has been described.[36] Approximately 10% of albumin is glycosylated at lysine ε-amino groups in the serum of normal individuals. The level of glycosylation in diabetics has yet to be reported, nor is the significance of this process in terms of the transport and other functions of albumin known.

It should be pointed out that nonenzymatic glycosylation is not unique to the hyperglycemic state; rather, it simply occurs at a faster rate the more glucose is present. Thus, if protein turnover were slow enough, certain sequelae of diabetes might develop in non-diabetic individuals over a longer time course. For example, senile cataracts might also be caused by glycosylation of lens crystallins. In fact nonenzymatic glycosylation may play a major role in the normal aging process, since the complications of diabetes are frequently considered to resemble accelerated aging.

Furthermore, proteins may be modified by carbohydrates other than glucose. A galactosylation of lysine residues has also recently been implicated in the genesis of cataracts of rats fed a high galactose diet.[37]

Summary - The tendency of sugars and amines to condense to form stable Amadori rearrangement products was recognized decades ago as an important reaction in the food and agricultural industry. Indeed, the Amadori rearrangement is the first step in the Maillard reaction, or nonenzymatic browning of food.[38] Only recently, however, has the role of nonenzymatic glycosylation in human biology come under investigation.

Hemoglobin was the first protein shown to be modified in this manner. Glucose reacts to form a Schiff base with NH_2-terminus of the β chain of Hb A, and subsequently undergoes an Amadori rearrangement to yield 1-amino-1-deoxyfructose. This modified hemoglobin, named Hb A_{Ic}, is a normal red cell constituant present in increased concentration in patients with diabetes mellitus. Hb A_{Ic} is synthesized throughout the life of the erythrocyte in a slow, nearly irreversible reaction. The rate of synthesis is a function of blood glucose concentration. These properties of Hb A_{Ic} combine to make it an indicator molecule whose concentration at one point in time reflects the patient's mean blood glucose level for the preceding month. This gives Hb A_{Ic} a unique and invaluable role in clinical medicine, for it is the only known parameter that accurately assesses long term carbohydrate control.

While Hb A_{Ic} itself is not likely to have deleterious effects, the nonenzymatic glycosylation of other proteins may result in altered enzymatic activity, solubility, antigenicity etc. and thereby results in many of the clinical sequelae of long-standing diabetes. In this regard, lens proteins undergo increased glycosylation in high carbohydrate environments, and such glycosylation increases the ease with which the proteins oxidize to form high molecular weight aggregates and opacities. Similar glycosylation reactions should occur at a slower rate in normal individuals and may account for many of the changes seen in normal aging, e.g., senile cataracts.

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Section VI - Topics in Chemistry and Drug Design

Editor: Burt Renfroe, CIBA-GEIGY Corporation, Ardsley, N.Y. 10502

Chapter 26. Reactions of Interest in Medicinal Chemistry

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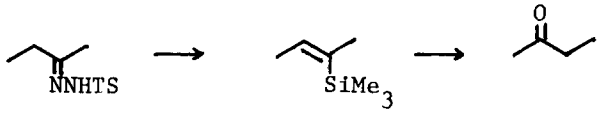
The selection of novel reactions deemed potentially useful to medicinal chemists from the great mass of this past years' literature in organic chemistry obviously involves a highly personal view of the field. Some of the seventy odd transforms which follow were picked because they seem to offer more convenient access to important functional arrays, because they lead to moieties which may be associated with biological activity or simply because they involve clever chemistry.

REVIEWS - Useful reviews have appeared entitled: "Asymmetric Syntheses,"[1,2] "New applications of Malononitrile in Organic Synthesis,"[3,4] "Intramolecular Ene Reaction in Organic Synthesis,"[5] and "α-Sulfenylated Carbonyl Compounds in Organic Synthesis."[6] Additional articles summarize stereospecific olefin syntheses,[7] heterocyclic syntheses by benzyne cyclizations,[8] and ring transformations of pyrimidines.[9]

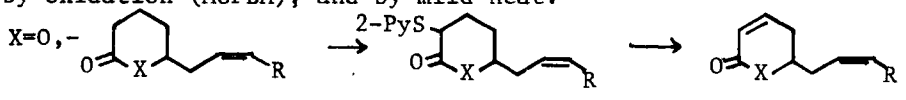
REAGENTS - *Esterification* - Carboxylic acids can be esterified in high yield under very mild conditions by a new procedure using DCC and 4-(N-pyrrolidino)pyridine. The procedure gives good yields even with mesitoic acids or with tertiary-butanol.[10]

Oxidations - 3,5-Dinitroperbenzoic acid is a stable storable peracid equivalent in activity to trifluoroperacetic acid.[11] A full paper has appeared which gives the experimental details for the α-hydroxylation of carbonyl compounds by treatment of the anions of enol silanes with MoO_5•HMPA (MOOPH).[12] Anions from carboxylic esters ($LiN(iPr)_2$,LDA; -78°C) can be efficiently, regiospecifically chlorinated or brominated by treatment with respectively CCl_4 or CBr_4.[13] Treatment of enol silanes from conjugated ketones with m-chloroperbenzoic acid (MCPBA) followed by removal of silicon affords the α-hydroxyketones.[14]

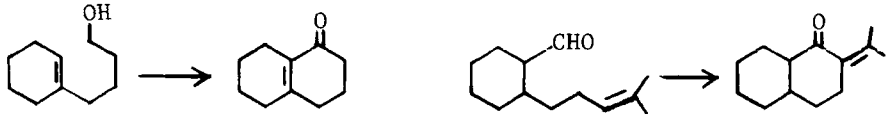
A new method for transposition of carbonyl groups starts by interception of the intermediate from tosyl hydrazone decomposition with Me_3SiCl. Oxidation of the silane intermediate with MCPBA gives the transposed product.[15]



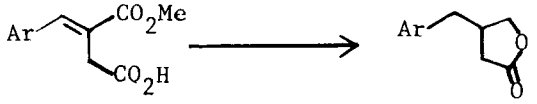
Cyclohexanones can be dehydrogenated to enones by treatment of the corresponding enol silanes with DDQ in the presence of a catalytic amount of the bis-silyl derivative of acetamide. However, the yields fall off drastically when the reaction is applied to cyclopentanones and cycloheptanones.[16] Another new method for dehydrogenation of carbonyl compounds consists of conversion to their pyridine-2-sulfide derivatives, followed by oxidation (MCPBA), and by mild heat.[17]



Oxidation of primary alcohols carrying unsaturation at the 5 or 6 position by means of the pyridinium chlorochromate provides the cyclized products. The same products are obtained by oxidation of analogously substituted aldehydes, with this reagent.[18]

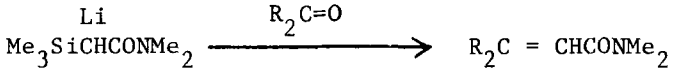


Reductions - Azides are efficiently reduced to the corresponding amines by 1,3-propylenedithiol.[19] Calcium borohydride (from $CaCl_2$ and $NaBH_4$) has been used to reduce unsaturated glutaric monoesters directly to the lactones.[20]

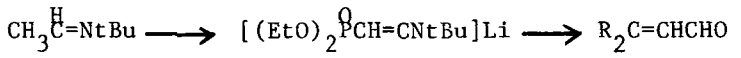


Condensations - Methyl ketones are converted to the corresponding vinyl compounds in high yield by treatment with paraformaldehyde in the presence of N-methylaniline trifluoroacetate in THF or dioxane.[21] The formamide of 2-(N-methyl)aminopyridine has been found to be an efficient reagent for the transfer of formyl groups to Grignard reagents.[22]

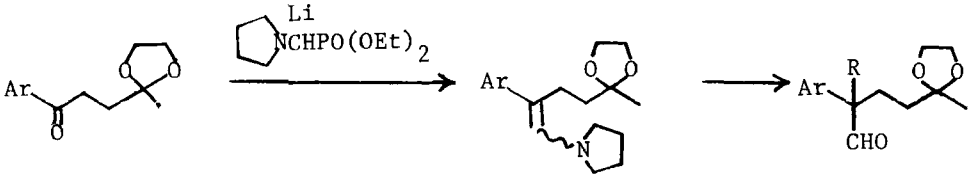
The salt obtained from treatment of 2-trimethylsilyl-N,N-dimethyl acetamide with LDA or BuLi is a stable solid. This gives good yields of acrylamides on reaction with ketones or non-enolizable aldehydes.[23]



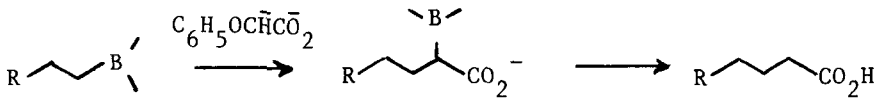
Reaction of the *tertiary*-butylimine from acetaldehyde with $(EtO)_2$-POCl, in the presence of two equivalents of base gives the corresponding ylide. This gives good yields of substituted acroleins on condensation with ketones and aldehydes.[24]



In a somewhat similar sequence, condensation of the functional ylide below with a ketone leads directly to an enamine. In the specific example at hand the crude product was alkylated without isolation. Hydrolysis of the ketal gives a cyclohexenone *via* the intermediate ketoaldehyde.[25]

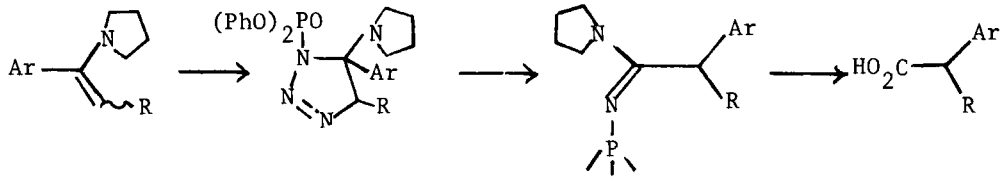


Organoboranes can be efficiently bis-homologated to the corresponding acetic acid derivatives by reaction with the dianion derived from phenoxyacetic acid. The stability of the phenoxide leaving group provides the driving force for the transformation.[26]

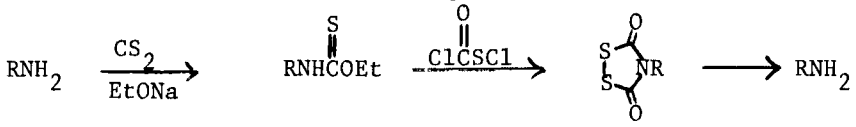


Miscellaneous - Enol chlorides can be converted to the corresponding ketones in fair yields at room temperature by inclusion of $Hg(OAc)_2$ in the reaction mixture.[27] A full paper has appeared which gives experimental details for the conversion of olefins to aminoalcohols by means of osmium reagents.[28]

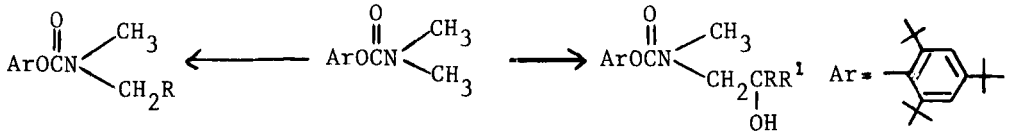
The side chain characteristic of nonsteroidal anti-inflammatory agents can be efficiently constructed by a novel rearrangement. Treatment of the enamine of aryl ketones with $(C_6H_5O)_2PON_3$ leads to the amidine derivative presumably *via* the triazole. Base hydrolysis gives the desired acid.[29]



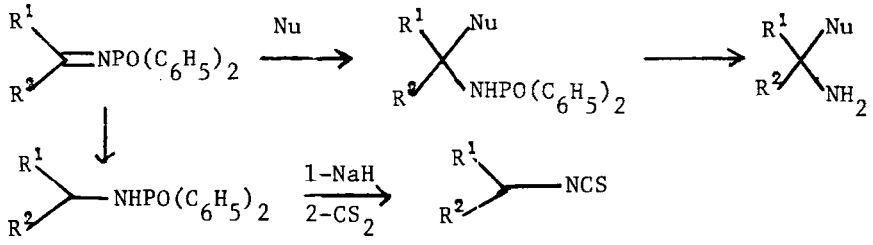
PROTECTING GROUPS - Cyclic imide disulfides from primary amines can be prepared by the scheme below. Treatment with mercaptoethanol under very mild conditions gives back the starting amine.[30]



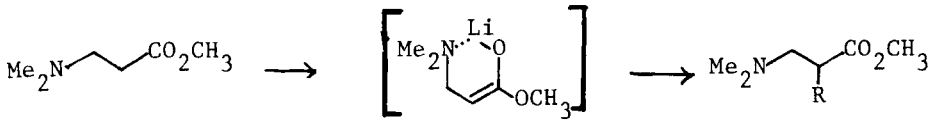
Acylation of dimethylamine with a hindered phenylcarbonate allows anion chemistry on the methyl groups.[31]



Phosphorus derivatives of imines show reactivity akin to ternary iminium salts, and thus readily add nucleophiles, (CH_3MgX;HCN). Isothiocyanates can be obtained under relatively mild conditions, by reduction followed by base and CS_2.[32]

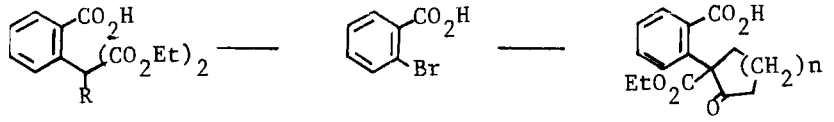


Addition of dimethylamine to acrylates protects the double bond and makes the position α to the carboxyl accessible to alkylation. Quaternization of the amine followed by treatment with base (DBU) regenerates a substituted acrylate.[33]

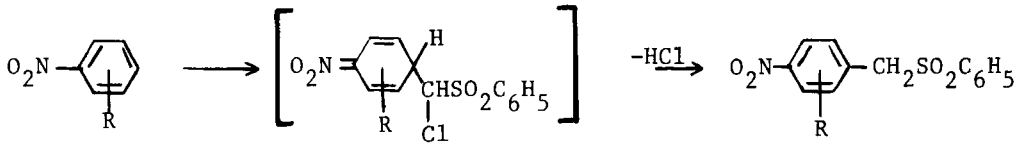


AROMATIC SUBSTITUTION - Reaction of oxazolines from o-fluorobenzoic acids with organometallic reagents results in net displacement of halogen. Hydrolysis of the heterocycle affords the substituted benzoic acid.[34]

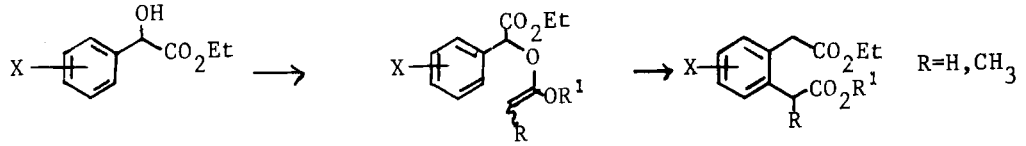
Condensation of enolates derived from β-dicarbonyl compounds with o-bromobenzoic acid in the presence of cuprous bromide affords the displacement product directly.[35]



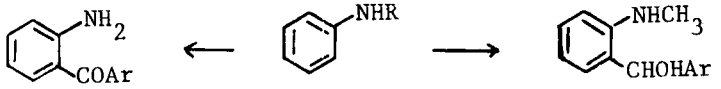
The driving force presumably lies in the stability of the halide leaving group. Formal replacement of hydride is possible with an appropriate nucleophile. Reaction of nitrobenzene with the anion from chloromethylphenyl sulfone gives the substitution product in 72% yield as a 1:1 *ortho* to *para* mixture. Use of bulkier anions gives exclusively *para* product. When the *para* position is substituted, the nucleophile enters *ortho*.[36]



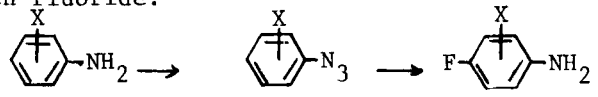
The Claisen rearrangement has only recently been used to provide functionalized derivatives. Thus, mandelate esters react with orthoacetates and orthopropionates to give the corresponding homophthalates.[37]



Condensation of the boron trichloride complex of N-methylanilines with aromatic aldehydes affords o-aminobenzhydrols in moderate yields. Somewhat lower yields of the corresponding ketones are obtained by condensing the adduct obtained from aniline itself with nitriles in the presence of $AlCl_3$.[38]

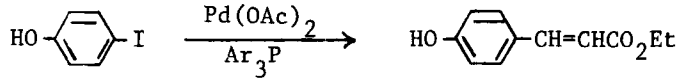


A very mild method for fluorination of the *para* position of anilines involves formation of the azide *via* the diazonium salt followed by treatment with hydrogen fluoride.[39]

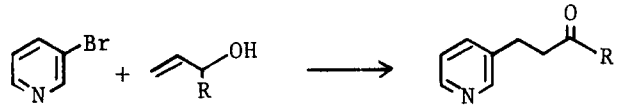


Aromatic iodophenols can be vinylated by ethyl acrylate in the presence of a palladium catalyst.[40] In an extension of this work the catalyst

was used to vinylate 2-bromothiophene and 3-bromopyridine.[41]

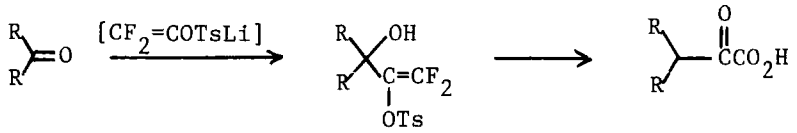


Reaction of 3-bromopyridine with allyl alcohols under similar conditions affords carbonyl compounds which result from migration of the double bond.[42]

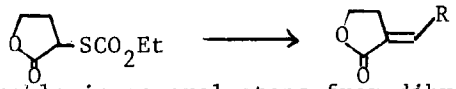


Mono-and dialkoxy-N,N-dialkylbenzamides can be lithiated *ortho* to the amide group by means of *sec*.BuLi in the presence of $Me_2NCH_2CH_2NMe_2$. In those cases where the alkoxy and amide groups are disposed 1,3, metallation occurs at the 2-positions. These organolithium reagents react with a variety of electrophiles.[43]

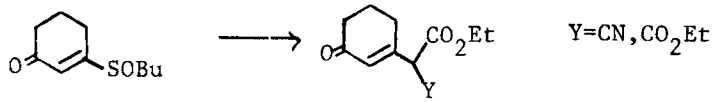
FORMATION OF CARBON-CARBON BONDS - The anion derived from the tosylate of CF_3CH_2OH readily adds to ketones or nonenolizable aldehydes. Sequential treatment of the adduct with acid and base gives the substituted pyruvic acid.[44]



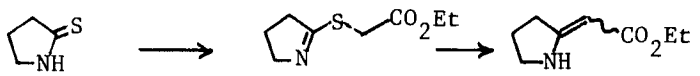
An interesting method for formation of α-vinylene lactones relies on sulfur for activation of the α-position. Condensation of the anion of the thiocarbonate with a variety of aldehydes gives olefins in moderate to excellent yields with high stereoselectivity.[45]



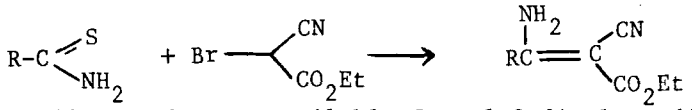
The sulfoxide obtainable in several steps from dihydroresorcinol adds the anion from diethyl malonate or ethyl cyanoacetate. The intermediate sulfoxide anion undergoes spontaneous elimination to afford unusually substituted cyclohexenones.[46]



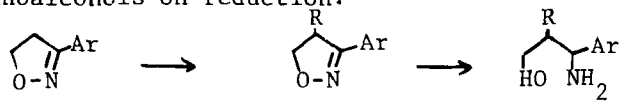
Alkylation of thiopyrrolidone with ethyl bromoacetate gives the thioether, which undergoes sulfur extrusion with carbon-carbon bond formation on treatment with $(C_6H_5)_3P$ and base.[47]



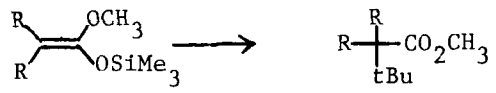
In an analogous reaction, open chain thioamides react with ethyl bromocyanoacetate (but not bromomalonate) to give the corresponding vinylidene derivatives.[48]



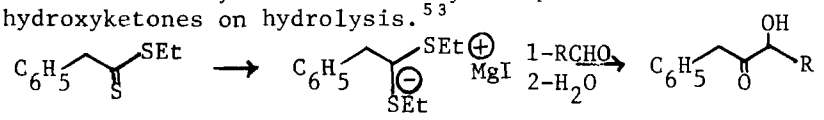
4-Arylisoxazolines, available from 1,3-dipolar addition of nitrile oxides to styrenes,[49] can be alkylated by means of LDA. These readily afford 1,3-aminoalcohols on reduction.[50]



Silyl enol ethers of carboxylic acid esters can be alkylated by means of $(CH_3)_3CCl$ in the presence of $ZnCl_2$ to give α-butylated products.[51] The reaction seems strangely immune to steric hinderance. Silyl enol ethers of ketones undergo an analogous transformation.[52]

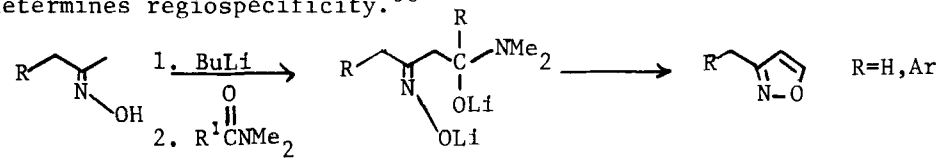


Thioesters add ethylmagnesium iodide to form a new organometallic reagent. This readily adds to carbonyl compounds to afford the corresponding hydroxyketones on hydrolysis.[53]

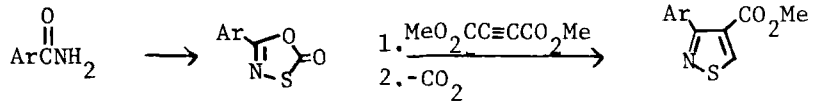


The anion from the Schiff base of cyclohexanone with optically active *tertiary* butyl α-aminoesters can be alkylated to afford products which are 73-97% optically pure.[54]

HETEROCYCLES - Isoxazoles can be formed regiospecifically in good yields from oximes by first acylating the dilithio salt with dialkylamides, followed by quenching of the adduct with H_2SO_4. Stereochemistry of the oxime determines regiospecificity.[55]



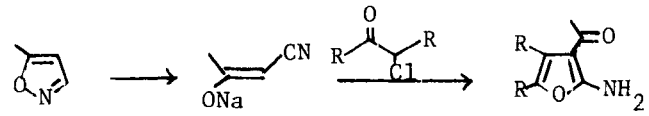
Isothiazole-4-carboxylates are obtained starting from amides by first forming the oxathiazolone by means of ClCOSCl. Condensation with methyl acetylene dicarboxylate followed by hydrolysis and decarboxylation completes the sequence.[56]



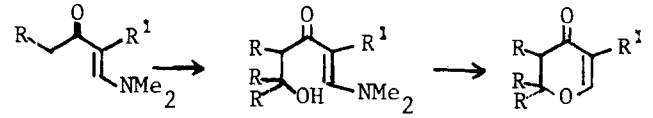
Aluminum chloride catalyzed condensation of nitriles with diazoketones affords good yields of oxazoles.[57]



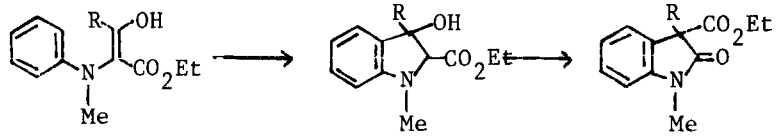
A general synthesis of 2-amino-3-acylfurans involves acylation of the enolate from cyanoacetone with chloroketones.[58]



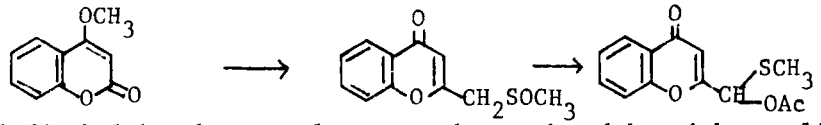
Condensation on the anion derived from the amino-enone with aldehydes or ketones gives the corresponding hydroxyketones. These close to dihydropyrones on treatment with MeOH•HCl.[59]



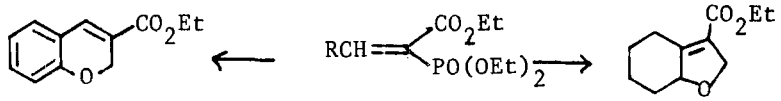
Condensation of N-methylaniline with terminally substituted α-bromo acetoacetates gives the corresponding acrylates. These can be cyclized photolytically to 3-hydroxyindolines. Treatment of the products with $Pb(OAc)_4$ leads to the rearranged indolones.[60]



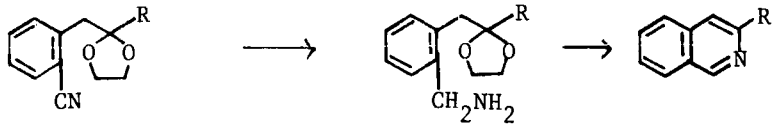
Addition of dimsyl anion to coumarones leads to substituted chromones. These undergo Pummerer rearrangement to the latent aldehydes.[61]



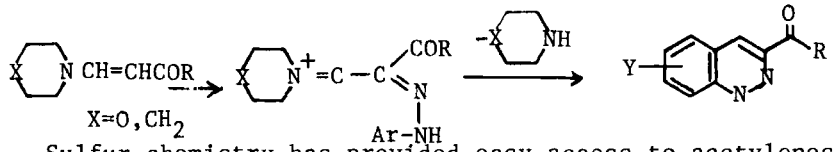
Ethyl diethylphosphonoacrylates can be produced by either sulfur[62] or selenium[63] chemistry. These react with salicylaldehydes to give dihydrobenzopyrones. Condensation with 2-hydroxycyclohexanone gives the hexahydrobenzofuran.[63]



A new synthesis for 2-substituted isoquinolines starts with o-toluonitrile. Base catalyzed acylation followed by ketalization gives the key intermediate. Reduction of the amine followed by acid cyclization and dehydrogenation (I_2) gives the heterocycle.[64]

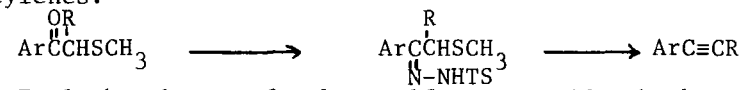


Aminoacrylates readily couplewith diazonium salts to form transient imines. These cyclize spontaneously to cinnolines.[65]

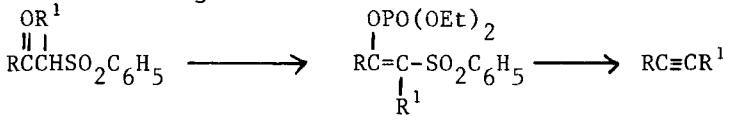


SYNTHONS - Sulfur chemistry has provided easy access to acetylenes. Re-

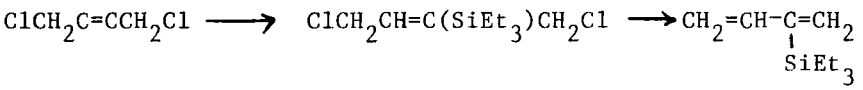
action of phenacylsulfides or their alkylation products with tosylhydrazine gives the corresponding hydrazones. Treatment with CH_3Li gives the acetylenes.[66]



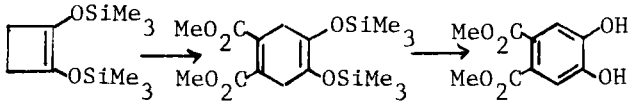
Enol phosphates of α-ketosulfones provide the key intermediate to alternate schemes leading to acetylenes. Acylation of phenyl sulfones with esters affords the ketone derivatives directly.[67] Alternately, the ketones can be obtained by Moffat oxidation of the condensation products from aldehydes with sulfone anions.[68] Conversion to the enol derivatives followed by treatment with Na/NH_3 or Na(Hg) gives the acetylenes.



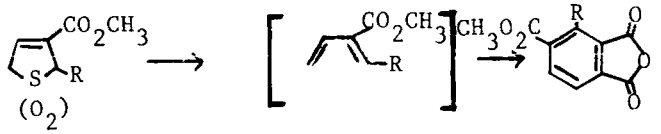
The recent development of dienes incorporating latent functionality has greatly expanded the utility of Diels Alder approaches to synthesis of complex molecules. Butadiene substituted by silicon at the 2-position is available from 1,4-dichloro-2-butyne by H_2PtCl_2 catalyzed reaction with Et_3SiH followed by dehydrohalogenation (Zn). The product reacts readily with dienophiles.[69]



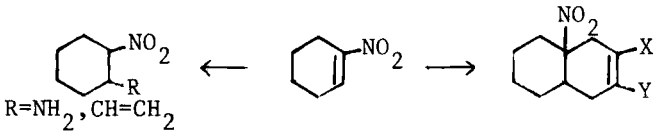
Pyrolytic scission of cyclobutenes leads to a diene which promises to be useful in a Diels Alder approach to catechols. Heating of the silyl derivative of the acyloin product from dimethyl glutarate in the presence of dienophiles affords adducts of the transient diene. The adduct obtained with dimethyl acetylene dicarboxylate was converted in several steps to phthalic acid derivative.[70]



A general procedure has been developed for the preparation of 1,4-dihydrothiophene-3-carboxylates starting from acetaldehyde-2-thiol.[71] The corresponding sulfones extrude SO_2 on refluxing in toluene to give the substituted dienes. These readily condense with maleic anhydride.[72]

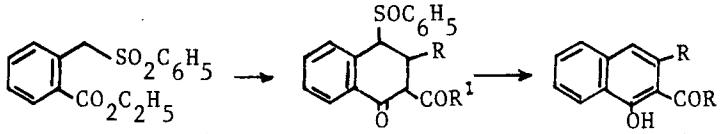


Nitromercuration of olefins followed by base catalyzed elimination of mercury gives the product of olefinic nitration. 1-Nitrocyclohexene obtained by this scheme readily undergoes conjugate addition of nucleophiles and serves as a dienophile.[73]



Monoacetals of quinones are accessible by $Tl(NO_3)_3$ oxidation of hydroquinone monomethyl ethers. The unprotected carbonyl group can be condensed with anions to afford products which give substituted phenylacetic acid derivatives on aromatization.[74]

A versatile non-Friedel-Crafts approach to naphthols is provided by condensation of the toluic acid sulfoxide with substituted acrylates or methyl vinyl ketone. Sequential conjugate addition and acylation of the first formed anion leads to the tetralone. Pyrolysis leads to aromatization by loss of sulfinic acid.[75]



A related scheme which leads to hydroxytetralones involves reaction of the anion from a phthalide (LDA) with an unsaturated ester. This reaction too involves a conjugate addition-acylation sequence.[76]

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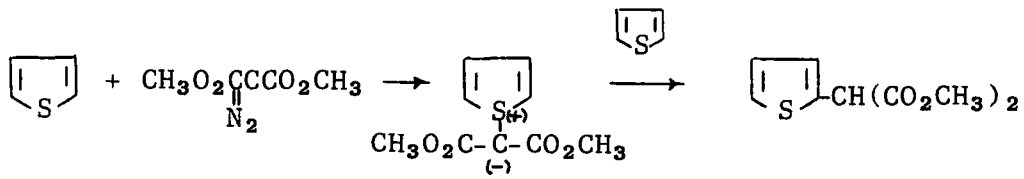
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Chapter 27. New Methods in Heterocyclic Chemistry

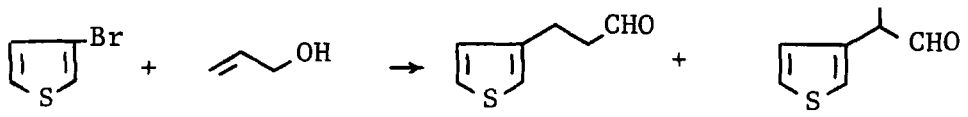
Edward C. Taylor
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Introduction - The immensity of the literature in heterocyclic chemistry precludes a comprehensive treatment in the few pages allocated to this chapter. As a consequence, we survey this year only three areas of synthetic interest - the introduction and modification of substituent groups, new methods for ring annelation, and synthetically useful ring transformation reactions - and we will reserve for future years a discussion of recent advances in such areas as 1,3-dipolar cycloaddition reactions, applications of Diels-Alder reactions, Michael additions, and Wittig reagents to the construction of heterocycles, and topics such as N-oxide chemistry and the use of heterocycles as reagents in organic synthesis.

Substituent Group Chemistry - Numerous new methods for the introduction and modification of substituents continue to appear, reflecting the fact that this area of heterocyclic chemistry contains many of its greatest synthetic challenges. Pyrimidines may be brominated under neutral conditions by the use of nitronium tetrafluoroborate in sulfolane.[1] N-Substituted imidazole-2-carboxamides are readily formed by heating imidazole and 1-alkylimidazoles with isocyanates in nitrobenzene or diphenyl ether.[2] 2- and 4-Methoxyquinolines are converted into 2- and 4-bromo derivatives (replacement of $-OCH_3$ by -Br) under neutral conditions by heating at 60-80° with the complex formed from phosphorus tribromide and DMF.[3] An extension of the classical malonic ester synthesis with halo-substituted heterocycles involves the use of isopropylidene alkylmalonates in acetic anhydride; double decarboxylation of the products leads to alkyl substitution of the heterocycle.[4] A different concept has been applied to the synthesis of dimethyl 2-thienylmalonate; reaction of thiophene with dimethyl diazomalonate in the presence of Rh(II) acetate gave an intermediate ylid which was transformed into the desired product (an obvious intermediate to thiophene-2-acetic acid) on heating in excess thiophene.[5]

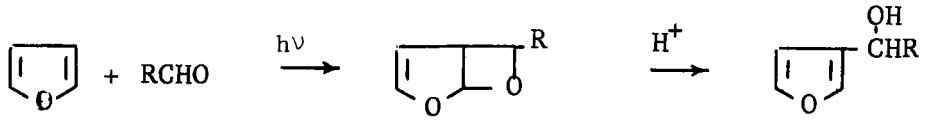


3-Substituted thiophenes (not readily available, since both electrophilic substitution and metallation take place at position 2) can be prepared from the readily available 3-bromothiophene by $Pd(OAc)_2$-catalyzed reaction with allylic alcohols.[6] This process has been extended to the preparation of 3-alkylpyridines from 3-bromopyridine,[7] as well as a variety of vinyl-substituted heterocycles from the corresponding bromo precursors.[8]



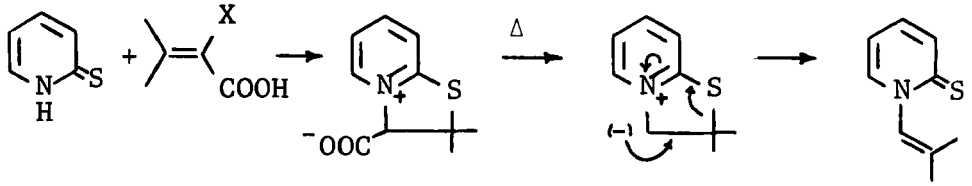
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3-Substituted furans, which are equally difficult to prepare by direct substitution into the furan nucleus, can be obtained by a variety of novel cyclization reactions,[9] or by acid-catalyzed isomerization of the cyclo-adducts (oxetanes) obtained by photoaddition of furan with aldehydes.[10,11]



Pyrimidyl aldehydes and ketones are conveniently prepared by the reaction of methyl-substituted derivatives with alkyl nitrites; the resulting aldoximes can be converted by hydrolysis to aldehydes, or by dehydration to nitriles, which can be further converted to ketones with Grignard reagents, or hydrolyzed and decarboxylated (replacement of $-CH_3$ by -H).[12,13] The introduction of substituent groups into protonated heterocyclic bases by free radicals, pioneered by Minisci and well reviewed over the past few years,[14-16] continues to be actively explored; further studies on alkylations,[17,18] acylations,[19] and carboethoxylations[20] have been reported. Various substituents (e.g., phenylsulfonyl, acetyl, phenylmercapto, halogen) are readily displaced by an ipso substitution process from the 2-position of benzothiazole by the nucleophilic 1-adamantyl and acetyl radicals.[21] Extension of these substituent group interchange processes to other heterocyclic systems could be of considerable synthetic utility.

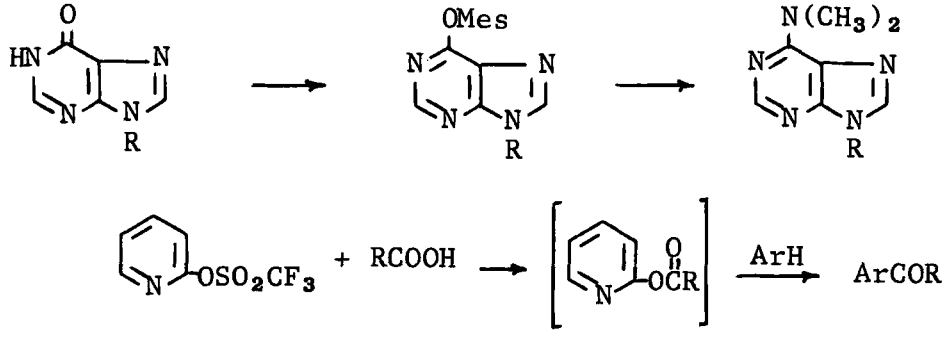
Although the classical method for the preparation of thiolactams involves reaction of lactams with P_2S_5 in high boiling solvents, a room temperature procedure involving addition of Et_3N to a suspension of P_2S_5 and the lactam in acetonitrile is effective even with sterically hindered secondary and tertiary amides.[22] Phase transfer catalysis has been recommended for specific S-alkylation of thiolactams,[23] while specific N-vinylation (of pyridine-2(1H)-thione) has been achieved by reaction with α-halo-α,β-unsaturated carbonyl compounds, followed by thermal ring opening (with loss of CO_2) of the resulting 3-carboxydihydrothiazolo(3,2-a)pyridinium derivatives.[24]



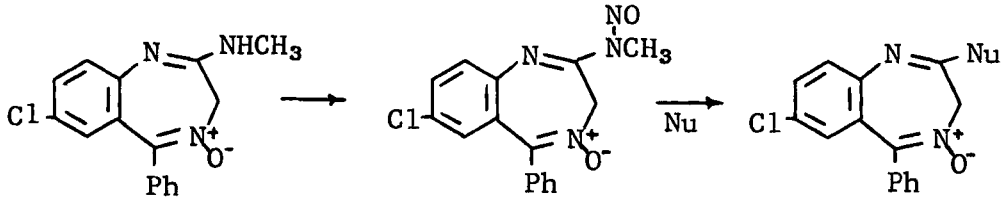
A general procedure for the introduction of substituents in heterocycles is displacement of a good leaving group by an appropriate nucleophile. The most reactive substituent towards displacement by nucleophiles appears to be methylsulfonyl (readily prepared by oxidation of a methylthio substituent); the trimethylammonium group is less reactive, but it is still some 700-1000 times a better leaving group than a chloro or a methylthio group.[25] Although alkylsulfonyl groups are more readily displaced than chloro groups by primary amines or ammonia, secondary and aromatic amines preferentially displace the latter (presumably because of steric hindrance due to a combination of the size both of the alkylsulfonyl group and the nucleophile).[26] Reaction products should be examined with care in such

systems, however, since migration of alkylsulfonyl groups under conditions of nucleophilic displacement occurs with some ease.[26-29] One must also be constantly alert to the intervention of cine or tele substitution processes (emergence of the attacking nucleophile at a position different from that occupied by the "displaced" leaving group), and to the ubiquitous ANRORC reactions in which ring-opening, ring-closure reactions can introduce many complications (exchange of endocyclic for exocyclic heteroatoms, Dimroth-type rearrangements, ring interconversions) into apparently straightforward "displacement" reactions.[30]

A substituent which appears to be even more readily displaced by nucleophiles than alkylsulfonyl groups is O-mesyl (OSO_2CH_3). Conversion of a cyclic lactam to its O-mesyl derivative is accomplished by treatment with methanesulfonyl chloride/Et_3N in methylene chloride solution; displacement takes place readily with dimethylamine (15 min at 20° in dioxane).[31] This may prove to be a generally useful procedure for replacement of lactam oxygen by other nucleophiles without resorting to intermediate chloro compounds. Note that 2-trifluoromethanesulfonyloxypyridine (from 2(1H)-pyridone, NaH, and trifluoromethanesulfonyl chloride) is an effective reagent for the facile acylation of activated aromatic compounds with carboxylic acids.[32]



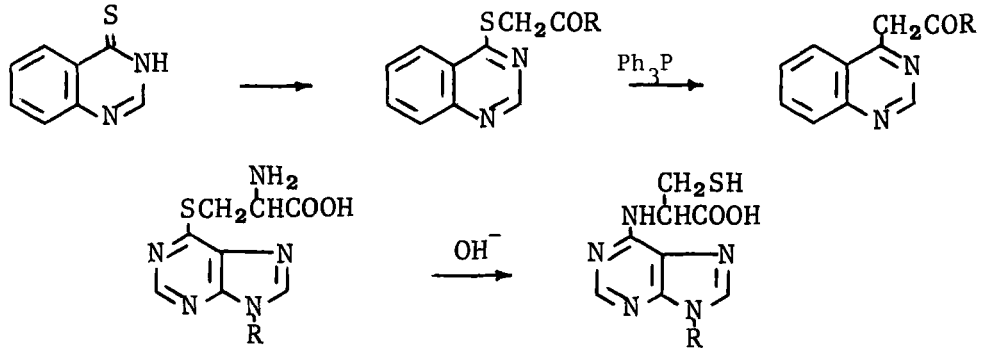
The methylamino grouping in Librium is easily displaced by nucleophiles by initial nitrosation to give the N-nitroso derivative, followed by reaction with methylhydrazine (loss of diazomethane); this procedure avoids the usual hydrolysis/chlorination sequence which would have affected the N-oxide functionality in the substrate.[33]



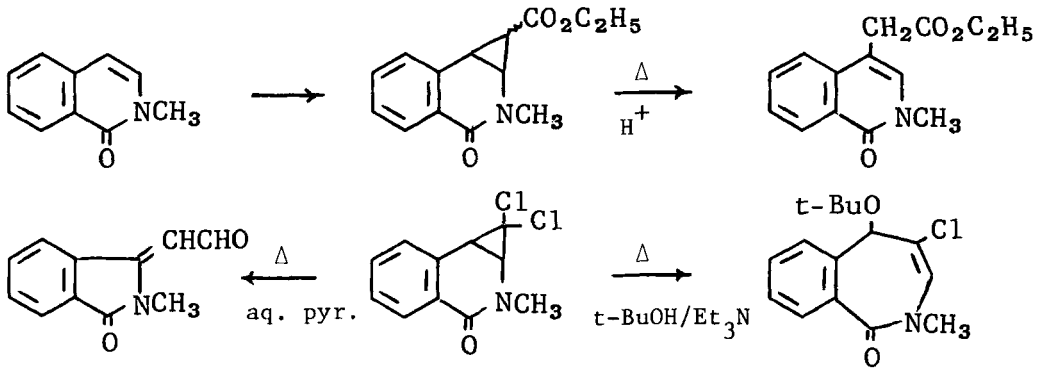
Some nucleophilic displacement reactions (particularly those involving ketone enolates and other carbon nucleophiles) proceed routinely in low yield, with poor material balance. There is increasing evidence that these reactions are actually radical chain processes catalyzed, in principle, by a single electron transfer from the anion to the heterocycle. In such cases

deliberate addition of a good one-electron transfer agent, or irradiation, might have a significant effect on the yield.[34]

An ingenious procedure for replacing sulfur in a thiolactam by a $-CH_2COR$ group involves alkylation of sulfur with an α-haloketone, followed by sulfur extrusion either with triphenyl phosphine or by heating in DMF (with or without added sodium ethoxide). The reaction has been successfully applied thus far to isoquinoline-1(2H)-thione,[35] quinazoline-4(3H)-thione,[36] and 6-mercaptopurine.[37] A closely related intramolecular sulfur displacement reaction with intriguing synthetic potential is illustrated by the conversion of the S-alkylation product from 6-chloropurine ribonucleoside and cysteine to the isomeric N-alkylated product by treatment with alkali.[38]

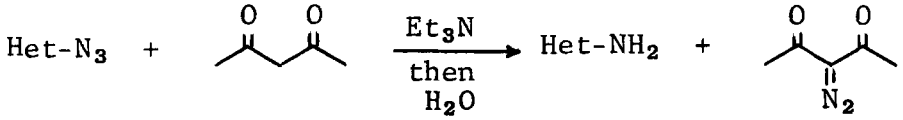


An efficient two-step procedure for the introduction of an acetic acid substituent into the 4-position of 2-methyl-1(2H)-isoquinolinone involves reaction with ethyl diazoacetate in the presence of a copper catalyst to give a mixture of exo and endo adducts, which are then isomerized to 4-carboethoxymethyl-N-methyl-2(1H)-isoquinolinone[39] with ethanolic HCl. Uracil 5-acetic acids could be prepared analogously.[40] The cyclopropane adduct from 2-methyl-1(2H)-isoquinolinone and dichlorocarbene can be ring-contracted to an isoindole, or ring-expanded to a benzazepinone.[40] The reaction of carbenes with other heterocyclic enamides has been reviewed.



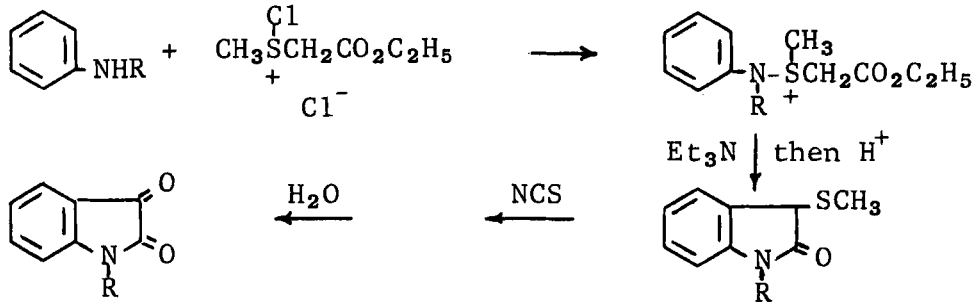
A selective procedure for the reduction of substituent azido groups (available by displacement of leaving groups by azide anion) to amino groups, without concomitant reduction of other reducible groups, involves an aza-

transfer reaction with acetylacetone.[41]

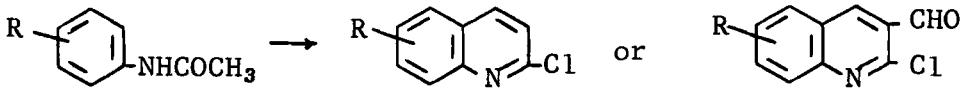


A versatile procedure for the introduction of alkyl,[42] alkenyl,[43] and epoxyalkyl[43] groups into heterocycles, which involves displacement of leaving groups by Wittig reagents, followed by appropriate manipulation of the resulting ylides, has been applied to the synthesis of various 6-substituted purines designed as inhibitors of adenylsuccinate synthetase and lyase.[44]

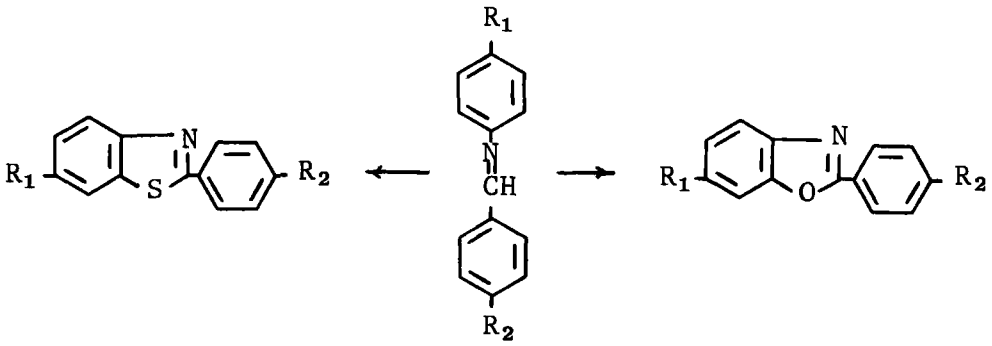
Ring Annelation Reactions - Many recent developments in synthetic methodology in heterocyclic chemistry have dealt with new methods for annelation of a heterocyclic ring unto a pre-existing ring system. A procedure for exclusive ortho substitution of aromatic amines by (2,3)sigmatropic rearrangement of ylides derived from N-arylazasulfonium salts has been applied to the preparation of indoles, oxindoles, N-methyl-2(1H)-quinolone, 3,4-dihydro-N-methyl-2(1H)-quinolone, isatins, and various condensed indole derivatives.[45]



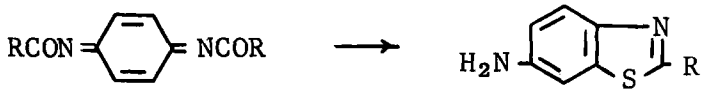
2-Diethylaminoquinolines are prepared in a single step from acetanilides and phenyl N,N,N',N'-tetraethylphosphorodiamidate (from $POCl_3$ by reaction first with phenol, and then with diethylamine).[46] Treatment of m-methoxy or m-methylacetanilides with the Vilsmeier reagent can be directed to give either 2-chloroquinolines or 2-chloro-3-formylquinolines; both are versatile intermediates for further substitution or ring annelation reactions.[47]



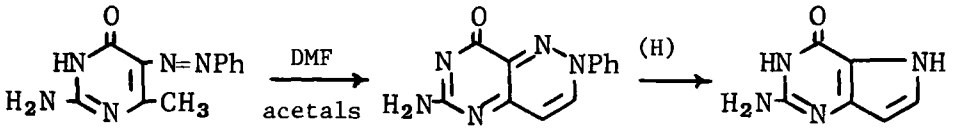
2-Arylbenzoxazoles and 2-arylbenzothiazoles are prepared by treatment of α,N-diarylnitrones with O-methyl diphenylphosphinothioate and phenylphosphonothioic dichloride respectively.[48]



Addition of thiolacetic acid to acylated p-benzoquinone imines (from oxidation of acylated p-phenylenediamines with $Pb(OAc)_4$), gives 6-aminobenzothiazoles.[49]

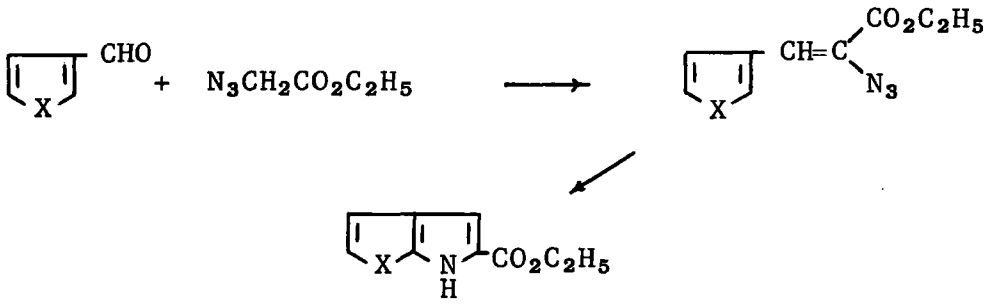


An ingenious procedure for the synthesis of 5H-pyrrolo(3,2-d)pyrimidines involves treatment of a 6-methyl-5-phenylazopyrimidine with t-butoxybis(dimethylamino)methane or an orthoformate ester, followed by hydrogenolytic ring contraction of the resulting pyrimido(4,3-c)pyridazine.[50]

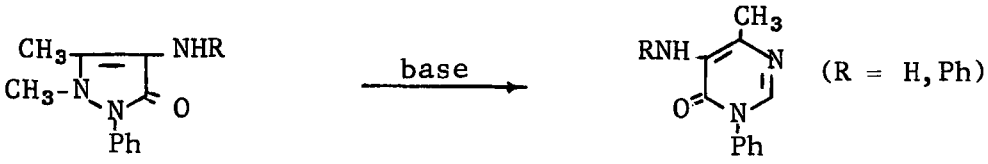


By contrast, treatment of 1,3-dimethyl-6-amino-5-arylazouracils with orthoformate esters,[51] followed by reduction with dithionite, yields 8-arylaminotheophyllines.[52] Many additional syntheses of condensed pyrimidines (pteridines,[53,54] azapteridines,[55] 5-deazaalloxazines,[56] 5-deazaflavins,[57] pyrimido(4,5-c)pyridazines[58]) involve treatment of other 6-amino and 6-hydrazinopyrimidine derivatives with formylating agents such as DMF acetal or orthoformate esters.

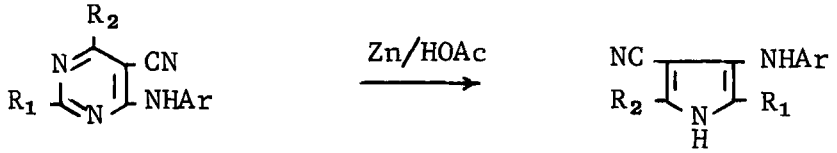
An alternate route for the annelation of a pyrrole ring employs the base-catalyzed condensation of furan-, thiophene-, and selenophene-carboxaldehydes with ethyl azidoacetate; the bicyclic products are indole isosteres.[59] The isomeric (3,2-b)pyrroles can be similarly prepared from the corresponding 2-carboxaldehydes.



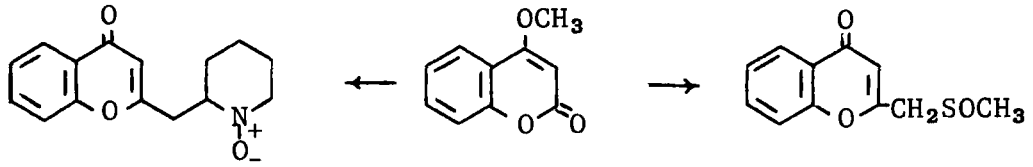
Synthetically Useful Ring Transformation Reactions - Many useful syntheses involve the deliberate transformation of one (presumably more readily available) heterocyclic system into another by a ring contraction, ring expansion, or ring transformation reaction. For example, 4-aminoantipyrines are readily expanded to 5-amino-4(3H)-pyrimidones with NaH, $NaNH_2$, NaOH or NaOEt in refluxing xylene.[59] By contrast, 2,6-disubstituted 4-arylamino-5-cyanopyrimi-



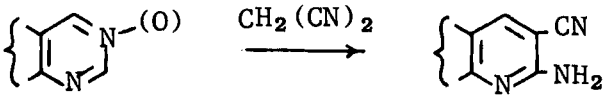
dines undergo ring contraction in high yield with zinc and acetic acid to give 2,5-disubstituted 3-arylamino-4-cyanopyrroles.[60]



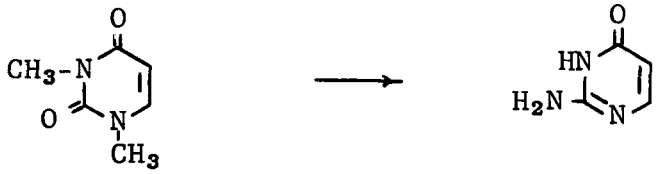
4-Methoxycoumarin reacts with a number of carbon nucleophiles via a ring opening, ring-closing sequence to give 2-substituted chromones.[61] The same



concept has been widely exploited for the conversion of pyrimidines to pyridines.[62]

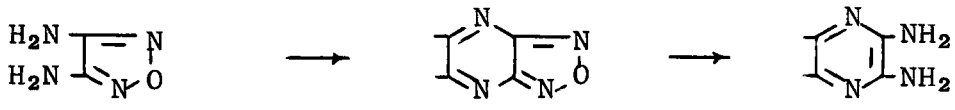


A novel pyrimidine-to-pyrimidine conversion exchanges N_1-C_2-N_3 of uracil derivatives for a N-C-N fragment of an attacking nucleophile; 1,3-dimethyluracil, for example, is converted to isocytosine with guanidine.[63] The reaction has unfortunately not yet been successfully applied to fused uracil derivatives.

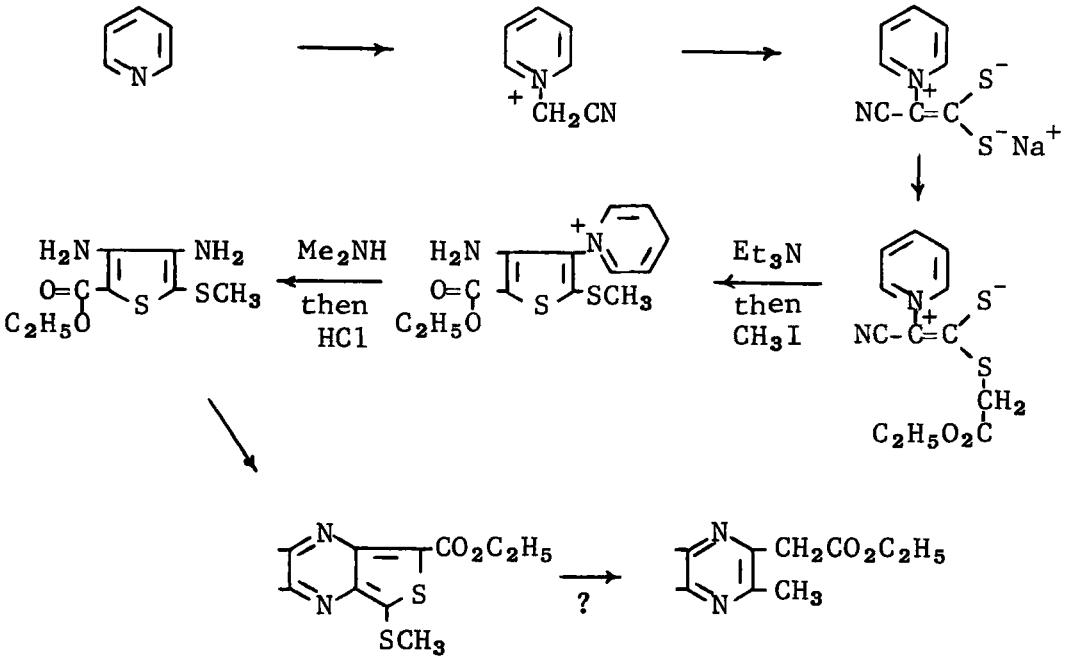


3,4-Diaminofurazan has been employed as a latent tetraaminoethylene in a synthesis of 2,3-diaminopyrazine;[64] reductive cleavage of the fused furazan ring extends a concept previously employed for the preparation of adenines,[65,66] pteridines,[67] and azapteridines,[67,68] of utilizing the furazan

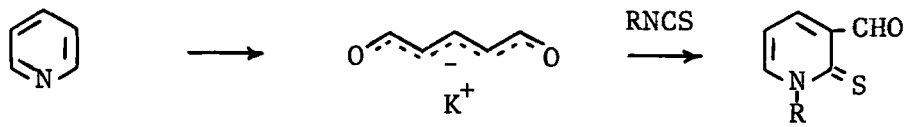
ring as a protected o-diamine functionality.



A related synthesis of fused pyrazines employs 3,4-diaminothiophenes (ingeniously prepared from pyridine as depicted below) as latent enediamines; condensation with 1,2-dicarbonyl compounds gives thieno(3,4-b)pyrazines which would appear to be potential intermediates to novel pyrazines by reductive desulfurization.[69]



The above example in which a pyridinium ring serves as a latent primary amino substituent is an extension of the classical "Zincke salt" procedure for transfer of the five carbon atoms of the pyridinium ring to an attacking nucleophile. A further example is the conversion of pyridine into 1-substituted 3-formyl-2(1H)-pyridinethiones by reaction of glutacondialdehyde (the hydrolytic cleavage product of the Zincke salt formed from pyridine) with isothiocyanates.[70]



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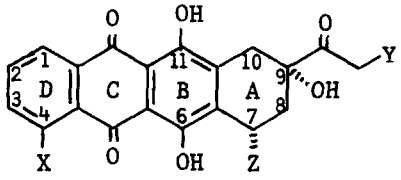
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Chapter 28. Synthetic Approaches to Anthracycline Antibiotics

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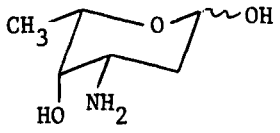
Introduction - During the past decade, the anthracyclines, most notably Adriamycin (1a, doxorubicin), daunomycin (2a, daunorubicin) and carminomycin (3a), have emerged as important chemotherapeutic agents for the treatment of a broad spectrum of human cancers.[1] While 1a, 2a, and 3a are presently produced by fermentation, the challenge of developing practical, totally synthetic routes to these molecules, coupled with the quest for superior analogs, has stimulated intense activity on the part of the synthetic community. More than a score of anthracyclines occur naturally,[1] but apart from early syntheses of the fully aromatic aglycones,[1] nearly all work to date has been conducted in the context of 1a-3a. Accordingly, this survey will focus primarily on efforts in this area, with emphasis being placed on general strategies rather than operational details. Of necessity, discussion of the preparation of analogs which are actually (or formally) derivable from 1a-3a is deemed beyond the scope of this review, as is the synthesis of analogs whose structures are only vaguely similar to 1a-3a.



a series (mycins), Z= L-daunosamine (α-anomer)

b series (mycinones), Z=OH

1, X=OCH_3, Y=OH
2, X=OCH_3, Y=H
3, X=OH, Y=H
4, X=Y=H
5, X=H, Y=OH



6, L-daunosamine

Like Gaul, the synthesis of the anthracyclines is divided into three parts: construction of the aglycones (e.g.,adriamycinone, 1b, daunomycinone, 2b, or carminomycinone, 3b), synthesis of the sugar residue 6 and coupling of the two.

Aglycone synthesis - The principal synthetic challenges posed by the aglycones include generation of the tetracyclic skeleton, introduction of the A-ring functionality and achievement of the "correct" (i.e., natural) regiochemical juxtaposition of the substituents in the A-and D-rings. The demonstration [2-4] that the natural, cis orientation of the 7-and 9-hydroxyl groups is thermodynamically preferred (∿6:1 ratio) and that epimerization of the C-7 position is effected by CF_3COOH has attenuated the need to address aglycone stereochemistry directly. The recent finding [5-8] that 4a and 5a, the 4-demethoxy analogs of 2a and 1a, are ca. ten times as potent as 1a and 2a (they are also more toxic) may well have diminished the practical need for finding an efficient solution to the problem of regiochemistry. Nonetheless, efforts to achieve regiochemically controlled routes have led, inter alia, to a number of new methods of anthraquinone synthesis. Although many of these solutions remain to be applied to the preparation of 1b-5b themselves, the underlying con-

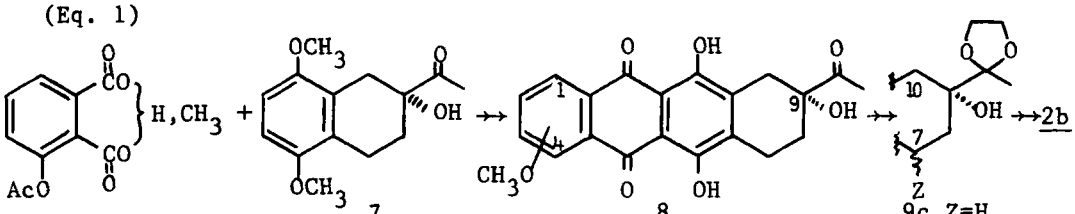
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cepts have become inextricably intertwined with anthracyclinone synthesis and will be included where appropriate. Interconversions among 1-5 have been developed. Introduction of the C-14 hydroxyl [2a→1a,[9] 2b→1b[4] (87%), 3(Y=H→Y=OH);[10] 4→5[11,12]] has been effected via the C-14 halo derivatives. Demethylation of 2b to 3b ($AlCl_3$, ~100%)[3,13] and methylation[14] of (+)-3b to (+)-2b (CH_2N_2, 45%) are recorded. Degradation of 1a to 2a[15,16] also permits preparation of ^{14}C-labelled 1 and 2.

In large measure syntheses of aglycones have relied on the employment of one (or more) of three general reaction classes: Friedel Crafts alkylations or acylations, nucleophilic condensations, and Diels Alder reactions;[17] the various routes are conveniently discussed under these three headings. (Note: unless otherwise indicated, all compounds described in the section on aglycone synthesis are racemic.)

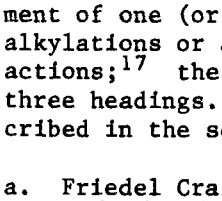
a. Friedel Crafts-type Routes - The first total synthesis of (±)-daunomycinone (2b) was reported in 1973 by Wong and coworkers (Eq.1),[2] and was

(Eq. 1)

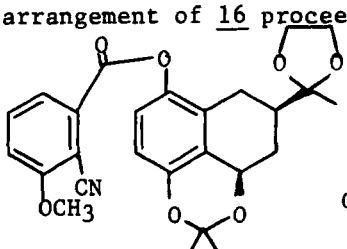


c series=4-OCH_3

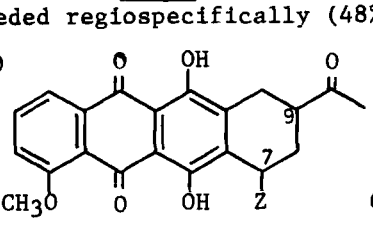
patterned after their earlier synthesis of 4(Z=OCH_3),[18] as well as model studies by Goodman and colleagues.[19] Although the original sequence(Eq.1) suffers from a low overall yield (since improved upon[14]) and lacks regiochemical control, the selective bromination (NBS) of C-7 (and not C-10—attributed to steric hindrance) and subsequent conversion to 7-hydroxyl have been used in numerous other routes. A refined version of this approach,[6,5] employing phthalic anhydride and (R)[20]-(-)-7, provides 7(S),9(S)-4b in 20% overall yield [based on (R)-7]. Analogs 11-15[6] (and the corresponding glycosides) have been similarly prepared. In an attempt to overcome the lack of regiochemical control in the Wong route, Kende et al.[21] prepared 16. Photo-Fries rearrangement of 16 proceeded regiospecifically (48% yield, based on recov-



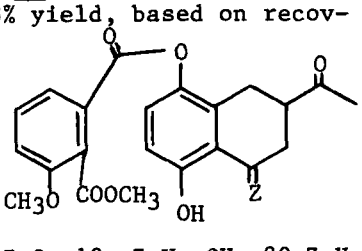
11,R=H,R'=CH_3
12,R=CH_3,R'=H
13,R=Cl,R'=H
14,R=H,R'=Cl
15,R=H,R'=-(CH=CH)$_2$-



16



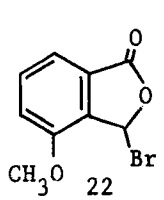
17, Z=OH, 21, Z=H

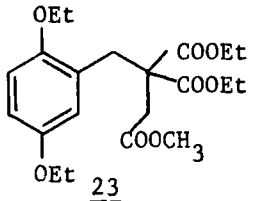


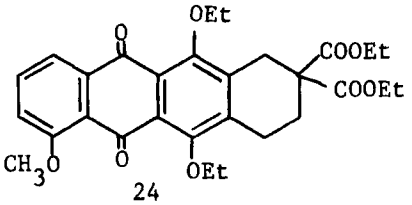
18, Z=O; 19, Z=H, OH; 20,Z=H_2

ery of 53% 16) to give, after further elaboration, 17. Introduction of the C-9 oxygen was not reported. A similar approach was examined by Sih

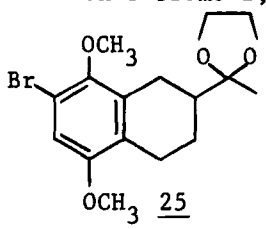
and coworkers[22] who prepared 18 regiospecifically by selective acylation of the non-hydrogen-bonded hydroxyl of the corresponding tetralone. Attempts to effect a Fries rearrangement and subsequent cyclization failed with 18 and 19. Tetracyclic material was achieved with 20, but regiospecificity was lost: a 3:2 mixture of 21 and its regiomer was produced in 37% yield. Conversion of 21 to 8c (50%) was accomplished by epoxidation of the enol acetate derived from 21. F. Johnson et al.[23] have recently developed a regiospecific synthesis of 21 in 20% overall yield from 22 and 23 via 24. The yield of (±)-2b from 21 (via 8c) is ca. 25%.[3,4,22]

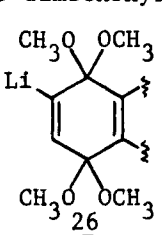

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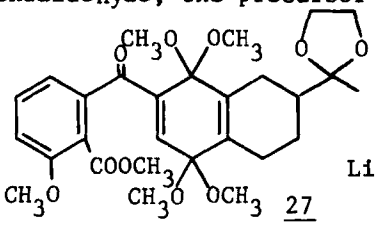

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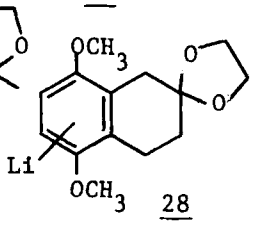

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b. Nucleophilic Routes - The search for effective solutions to aglycone regiochemistry has led to the development of a number of new methods for the construction of anthraquinones which involve anionic species in the regiochemically determining step. One of the more thoroughly implemented approaches is due to Swenton and associates.[24] Thus, 26, available in high yield from 25 in two steps (electrochemical methoxylation and transmetalation), reacts with dimethyl 3-methoxyphthalate to give 27 regiospecifically. Elaboration of 27 gives 21 in an overall yield of 14% based on 3-bromo-2,5-dimethoxybenzaldehyde, the precursor to 25. A re-

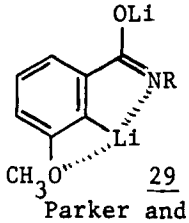

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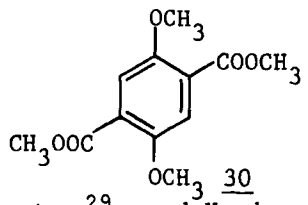

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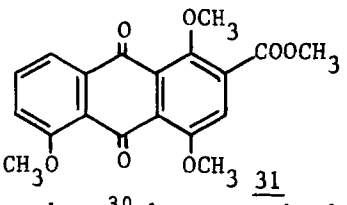

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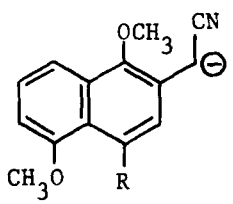
lated approach, which does not address regiochemistry, involves the reaction of 28 with dimethyl phthalate.[25] The use of ortho-lithiated benzamides for the regiospecific construction of anthraquinones (e.g. 29+30→→31) was reported virtually simultaneously from the laboratories of Baldwin,[26] Raphael,[27] and Snieckus,[28] but the methods remain to be extended to anthracycline synthesis.


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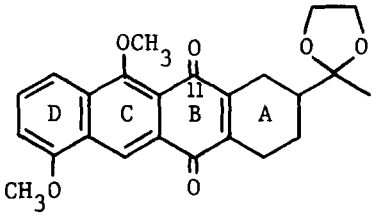

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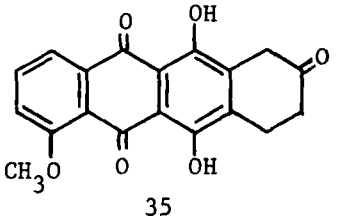
Parker and Kallmerten,[29] and Kende and coworkers[30] have examined the conjugate addition of 32 and 33 to a number of cyclohex-1-ene esters. Subsequent acylation of the pro C-ring with the ester moiety and hydroxylation α to the nitrile (the resulting cyanohydrin emerges upon decomposition as the C-11 oxygen) provide, in due course, 34 (from 32) and 35 (from 33) regiospecifically; 35 has been converted to (±)-daunomycinone



32,R=H;33,R=OCH_3

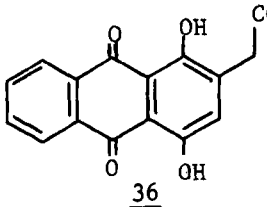


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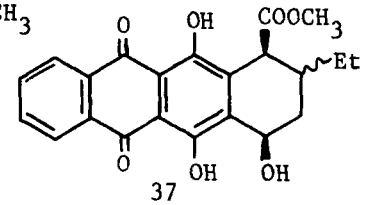


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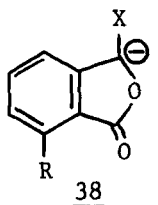
(2b) in ca. 20% yield (see below). A related reaction, that of 36 with pent-2-enal and NaH, provides the rhodomycinone analogs 37 in 50% yield.[32] The preparation of several rhodomycinones from 35 has also been described[86] Hauser and Rhee[33] and Kraus and Sujimoto[34] have utilized the conjugate addition of phthalide anions (38, X=CN or SO_2Ph) to unsaturated ketones for the preparation of naphthalene diols and anthraquinones[33] via 39.[35]



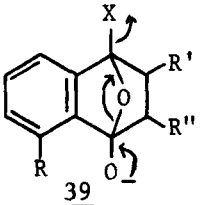
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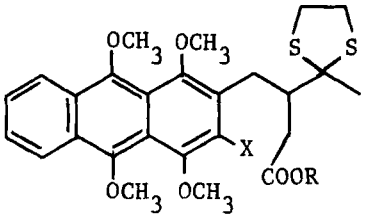


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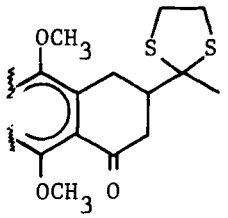


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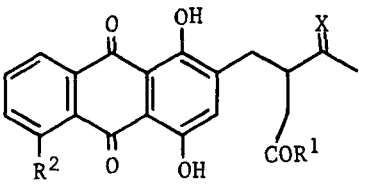
In a different approach,[36] treatment of 40 (R=H or CH_3) with n-C_4H_9Li gives 41 (55%) via 42; analogous treatment of the anthraquinone corresponding to 40 does not result in cyclization. While 41 is obtained quantitatively from 43 (R=H) with $(CF_3CO)_2O$,[36] neither acid nor base induces cyclization of 44 (R^2=H) or 45.[37] The less direct route involving base-catalyzed cyclization of the leuco derivative of 44 (R^2=H) gives 46a after aerial oxidation; the seemingly comparable conversion 44 (R^2=OCH_3)→leuco→



40,X=Br; 42,X=Li; 43,X=H



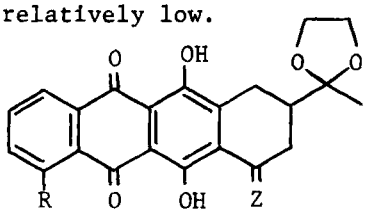
41



44,R^1=OCH_3, X=-OCH_2CH_2O-

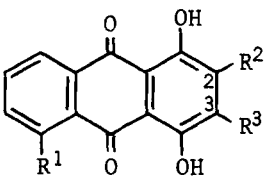
45,R^1=OH, R^2=OCH_3, X=O

47a virtually fails (<10%).[37] Intramolecular Marshalk alkylation of the aldehydes (R^1=H) derived from 44 (R^2=H and OCH_3) provides 46b and 47b,[37] which have been converted to (±)-2b and (±)-4b, but the overall yields are relatively low.



46,R=H; 47,R=OCH_3

a,Z=O; b,$Z=H_2$

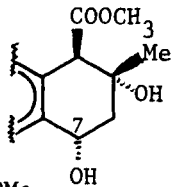


48,R^1=OH,R^2=R^3=H

49,R^1=OH, R^2=R^3=-$(CH_2)_4$-

50,R^1=OH,R^3=H R^2=-$(CH_2)_4OH$

51,R^1=H,R^2=CH_2COOMe R^3=$CH_2CH_2COCH_3$

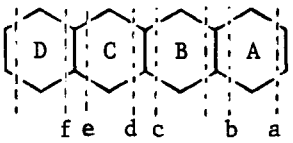


52

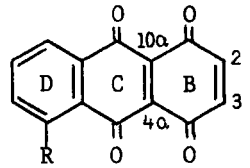
Sutherland and colleagues[38] have shown that Marshalk alkylation of 48 (which has been prepared on a 150kg scale)[39] with a variety of alde-

hydes can be directed to either the 2 or 3 position with high regioselectivity. The annulated derivative 49 can be prepared either directly from 48 (50%) by reaction with succindialdehyde[40] or from 50[38] (by oxidation and intramolecular Marshalk alkylation). The promise that this route holds for regioselective anthracyclinone synthesis is partly exemplified[41] by the Triton B-induced conversion of 51 (prepared by successive Marshalk alkylations of quinizarin) to 52 (26%), an analog of ε-rhodomycinone. The unusual hydroxylation at C-7 may involve a quinone methide.[42]

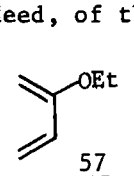
c. Diels Alder Routes - The power of the Diels Alder reaction for the expeditious construction of complex carbocycles has not escaped the notice of practitioners of anthracycline synthesis. Indeed, of the eight possible constructions implied by the vertical lines in 53, six (a-f) have appeared in at least one guise. Unfortunately, however, although Diels Alder routes have led to several efficient aglycone syntheses, they have not, except in one case (below), solved the regiochemical component of the problem. The most investigated route (53b), involves the quinizarin quinones 56. A serious complication to this approach is that many dienes (e.g. 57 and 58) add preferentially to the "wrong" double bond of 56.[3,44-48] Although several indirect solutions to this difficulty have been devised [protecting the C-4a,10a double bond of 56,[44,49] retaining the pro D-ring in latent form (59), use of quinizarin diboroacetate as a dienophile[50] (quinizarin itself does not exhibit dienophilicity)[51]], the most direct solution has been to manipulate the substituents in the diene to promote addition at C-2,3. At this writing three groups have developed dienes which meet this requirement and lead to complete syntheses of (±)-2b or (±)-4b. The first solution, which is also the first reported Diels Alder-based synthesis of (±)-daunomycinone (2b), is due to Kende et al.[3] It employs 2-acetoxybutadiene and 56b to give, after hydrolysis and tautomerization, 35 in 25% overall yield; the regioisomer is produced in similar amount. Reaction of 35 with HC≡CMgBr followed by hydration gives 8c (40%) which is hydroxylated at C-7 by an extension of the methods of Goodman and Wong (Br_2, hν; $AgOOCCF_3$; CF_3COOH) to give (±)-2b (50%). Conversion of 35 to 8c via 60, and thence to 2b was simultaneously reported by Henry et al.[4] When applied to 56a[12] the Kende sequence affords 61 in 36% overall yield; C-7 α-hydroxylation (45%) gives (±)-4b.[12] Reaction of 56a with 62 gives 63[48] (compare the reaction of 58) which is converted to 64 in four steps (40% from 56a). Surprisingly, attempts to hydrate the double bond of 64 to give (±)-4b directly have failed, but reduction (H_2,Pd/C) to 61 provides a less direct alternative. Chemists at Searle[46] have developed a synthesis of (±)-4b in 31% overall yield from 56a utilizing 65. Introduction of the C-9 substituents is effected via the ethy-



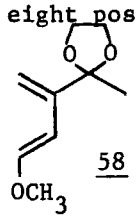
53



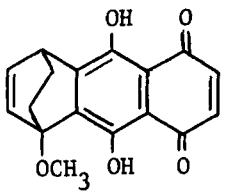
56a,R=H;56b,R=OCH_3



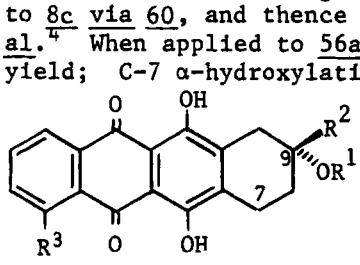
57



58

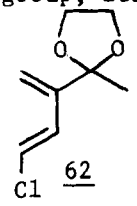


59

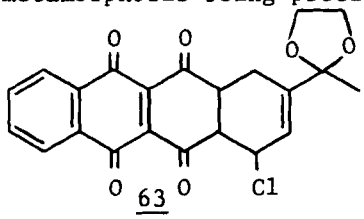


60,R^1=THP,R^2=CN,R^3=OMe
61,R^1=H,R^2=Ac,R^3=H

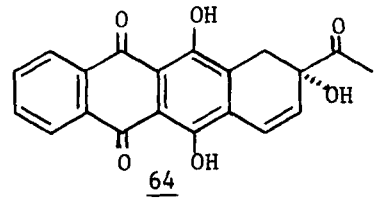
nylcarbinol. The trimethylsilyl group functions as a latent C-7 hydroxyl group, its metamorphosis being precipitated by $Pb(OAc)_4$ oxidation.



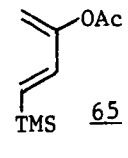
62



63

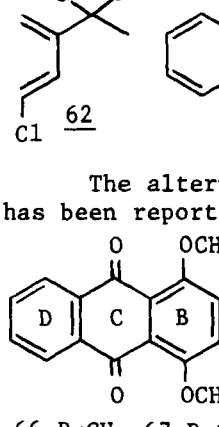


64

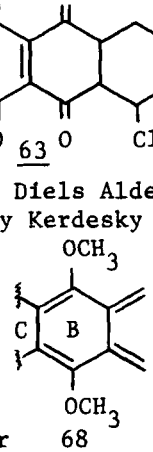


65

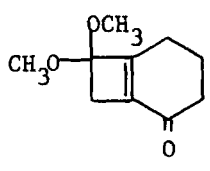
The alternate Diels Alder approach to A-ring construction (see 53a) has been reported by Kerdesky and Cava.[52] Thus 67, prepared via 66 in 73% yield from phthalic anhydride and 2,3-dimethylhydroquinone, upon treatment with zinc (→68) in the presence of methyl vinyl ketone gives the dimethyl ether of 9-deoxy-61 (52%). Hydroxylation at C-9 (t-BuOK, O_2, 55%) gives the dimethyl ether of 61 which has been converted to (±)-4b. The approach suggested by 53c has been examined by Boeckman et al.:[53] in situ generation (170°) of 69 from 70 and trapping with naphthoquinone gives 71 (75%) after oxidation; modifications to incorporate oxygen at C-6 have been unsuccessful. Jung and Lowe[54] have reported the regiospecific formation of 72 (~70% after oxidation) from the cycloaddition of 73 with juglone.[43]



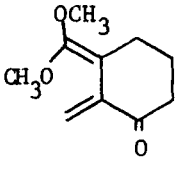
66, R=CH_3; 67, R=CH_2Br



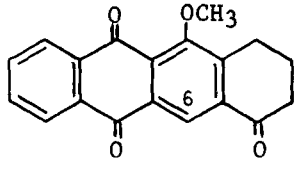
68



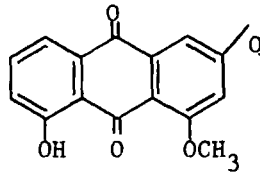
70



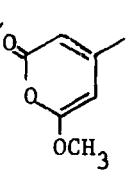
69



71

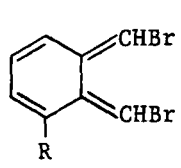


72

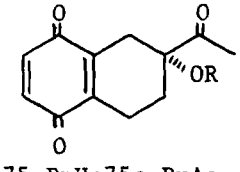


73

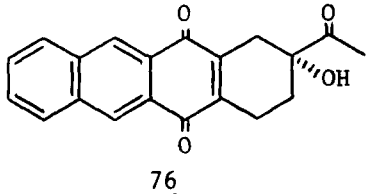
Several groups have investigated the route suggested by 53d. The most successful effort, that of Wiseman et al.[55] involves the trapping of 74(R=H) (from tetrabromo-o-xylene and zinc) with 75 (prepared in exceptional yield from the corresponding dimethoxytetralone) to give 76 (33%) which was converted to 61 in three steps (65%). Use of 74 (R=OCH_3) in the same sequence results in a mixture of regioisomers. Related approaches using 77 (X=H_2)[56] and (X=O)[57] have been reported, but have not been extended to a total synthesis.



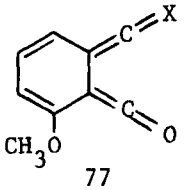
74



75, R=H; 75a, R=Ac



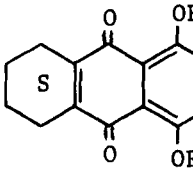
76



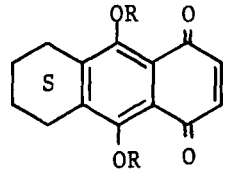
77

The salient finding of Fariña that substituted naphthazarins (e.g. 78a)[58] and their diacetates (e.g., 78b)[59] exist in tautomeric equilibrium (78⇄79) and that tautomers 79 can be selectively trapped by Diels Alder reactions was exploited by Kelly et al.[60] in the only regiospecific Diels Alder approach to anthracyclinones yet reported.[61] Thus, the asym-

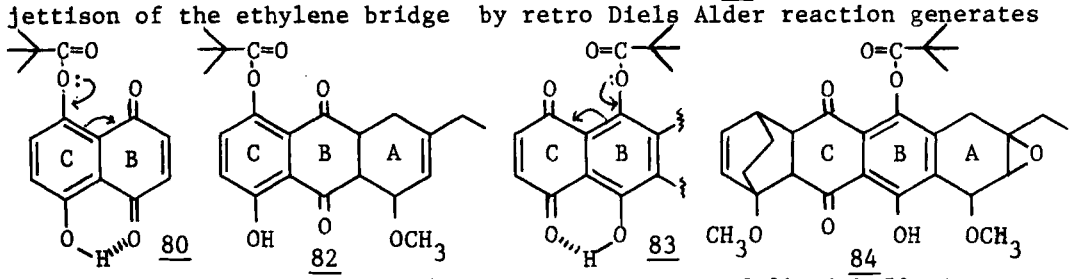
metry induced by the two "C"-ring substituents in 80 promotes regiospecific cycloaddition of 80 to 1-methoxy-3-ethylbutadiene (81), affording 82. After epoxidation and oxidation, tautomerization (with intramolecular transfer of the directing groups - see 83) permits a second, regiospecific Diels Alder reaction (with 1-methoxycyclohexa-1,3-diene), providing 84 in 45% yield based on 80. Oxidation and jettison of the ethylene bridge by retro Diels Alder reaction generates



78 a,R=H;b,R=Ac



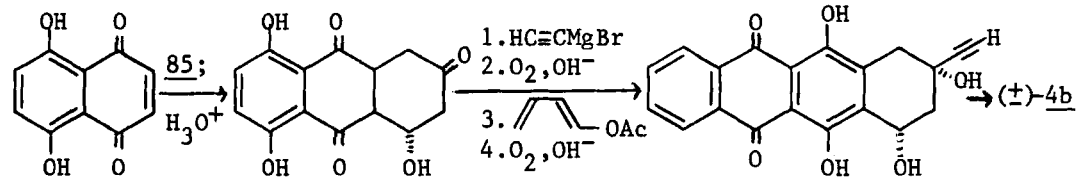
79



80 82 83 84

the aromatic D-ring. It is of note that reaction of 81 with 59 gives predominantly the "wrong" regioisomer.[51] A variation on this approach by

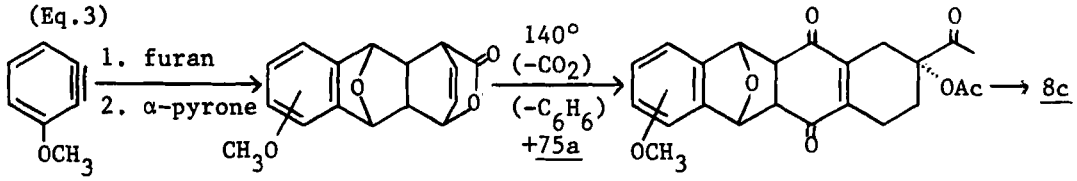
(Eq. 2)



Krohn and Tolkiehn[62] (Eq.2), which refrains from addressing regiochemical questions, employs 1,3-bis(trimethylsiloxy)butadiene (85) as an A-ring synthon and affords a notably efficient (29% overall yield) synthesis of (±)-4b with a virtually stereospecific introduction of A-ring functionality.

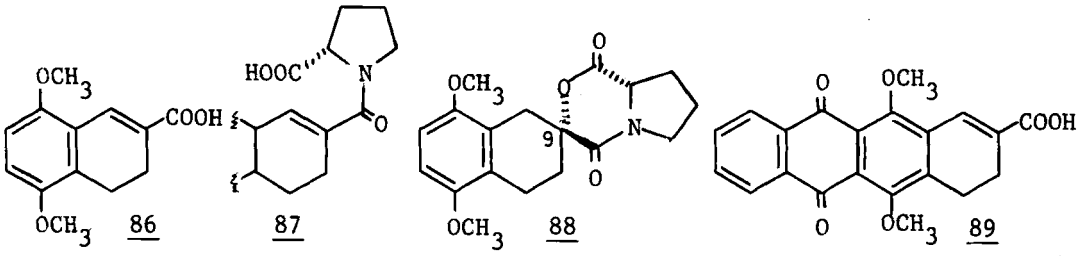
The unparalleled employ of five Diels Alder reactions (3 normal, 2 retro) in the Kende "isobenzofuran" route[12] (Eq.3) provides a fitting conclusion to the discussion of Diels Alder sequences. Although a 1:1 mixture of regioisomers is produced, the yield of 8c is 17% based on 75a.

(Eq.3)



d. Synthesis of Chiral Aglycones - If anthracyclines are to be available by total synthesis, the problem of aglycone chirality must be resolved. Resolution of racemic aglycones in the course of glycosidation is possible,[6] and classical resolutions of (±)-daunomycinone (2b)[3] and 7,[63] an intermediate common to several routes, have been attained. The possibility of developing asymmetrically induced syntheses of chiral aglycones remains, however, largely unexplored. Nonetheless, the only reported efforts in

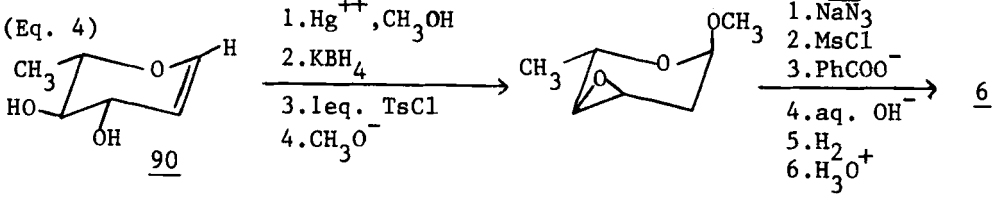
this area, those of Terashima and associates,[64] will be difficult to improve upon. Thus, conversion of 86 to the (-)-proline derivative 87, followed by bromolactonization and reductive dehalogenation provides a 98.5:1.5 mixture of 9-(R)- and 9-(S)-88. Further transformations (H_3O^+; CH_3Li) give (R)-7 in 44% overall yield from 86. The analogous "resolution" of 89 proceeds nearly as well.



86 87 88 89

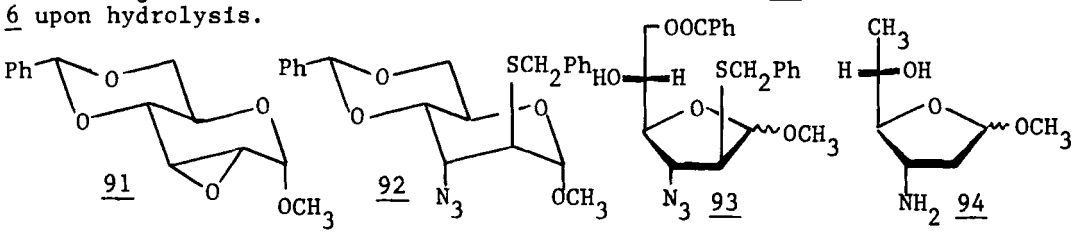
Syntheses of Daunosamine and its Isomers - Three syntheses of the natural L-antipode of daunosamine (6) have been recorded. The first, by Goodman and colleagues,[65] commences with L-rhamnal (90) and proceeds as shown in Eq.4. A second, due to Yamaguchi and Kojima[66] begins with 92 (from glu-

(Eq. 4)



90

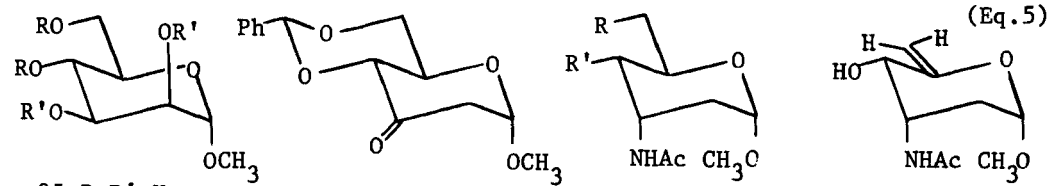
cose via the anhydroalloside 91) which upon methanolysis and benzoylation affords the furanoside 93 (41%, accompanied by a similar amount of the corresponding pyranoside). After conversion of 93 to the 5,6-epoxide (TsCl; CH_3O^-, 97%) reduction over Raney nickel gives 94 (36%) which yields 6 upon hydrolysis.



91 92 93 94

The most efficient synthesis of L-daunosamine is that of Horton and Weckerle;[67] it provides 6 (as its hydrochloride) in 40% yield from readily available methyl α-D-mannopyranoside (95). Thus butyllithium-induced cleavage[68] of the dibenzylidene acetal 96 affords 97 (91%), the oxime

(Eq.5)



95,R=R'=H
96,R=R'=PhCH

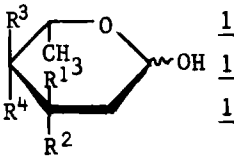
97

98,R=R'=$PhCHO_2$
99,R=Br,R'=PhCOO

100

(>95%) of which is reduced ($LiAlH_4$) and acetylated to give 98 (87%). After conversion to 100 (70%), stereospecific reduction and hydrolysis give 6.

Syntheses of the D-antipode of 6,[69-71] as well as the racemate,[72-73] have been described. Five of the remaining six configurational isomers (or their derivatives) of 6 have also succumbed to the labors of synthesis: L-*arabino* (101, acosamine),[5,74-78] D-*arabino*,[70] L-*ribo* (102, ristosamine),[5,79-81] D-*ribo*[82,83] and L-*xylo* (103);[71] the D-*xylo* isomer continues to be elusive. Glycosidation of 1b, 2b and 4b with 101 and 102 has been accomplished.[5]

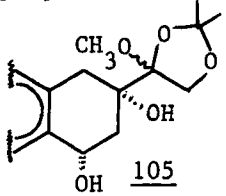


101, $R^2=NH_2$, $R^3=OH$, $R^1=R^4=H$

102, $R^1=NH_2$, $R^3=OH$, $R^2=R^4=H$

103, $R^1=NH_2$, $R^4=OH$, $R^2=R^3=H$

Glycosidation Methods - The coupling of 1b-5b with L-daunosamine (6) constitutes the final stage in total synthesis and has been effected using a variety of methods. It is necessary to protect the hydroxyl and amino substituents in 6 and, in the case of C-14 hydroxylated aglycones (e.g. 1b) to protect this functionality also: both the C-14 monomethoxytrityl ether (104)[4] and the dioxolane derivative 105[5,84] have been employed for this purpose (alternately, introduction of the C-14 hydroxyl can be deferred un-



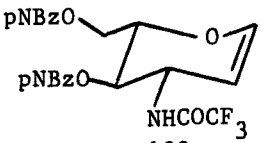
105



106, X=Br, R=pNBz

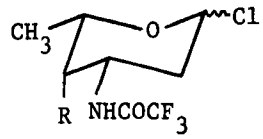
107, X=Cl, R=pNBz

108, X=α-Cl, R=$COCF_3$



109

til glycosidation has been carried out). The first preparation of 2a was reported in 1974:[85] coupling of 2b with 3 equiv. 106 under Koenigs-Knorr conditions gives the α-glycoside stereospecifically; deprotection gives 2a in 50% overall yield. Use of the more stable glycosyl chloride 107 raises the overall yield to 72% (still stereospecific), but again requires 3 equiv. of glycosyl halide.[4] Koenigs-Knorr coupling of 106 with 104 is also stereospecific and gives 1a in 40% overall from 1b, but the necessity of using 9 equiv. of 106 is a serious drawback.[4] The Koenigs-Knorr method has also been used for the preparation of a diverse selection of glycosides of ε-rhodomycinone, but yields and stereochemistry are variable.[87] Subtle changes in the exact structure of the glycosyl halide can have substantial stereochemical consequences:[5,6,84] use of 108 in place of 107 leads[84] to 1a (from 105) and 2a, but stereospecificity is lost in both cases, a 7:3 mixture of α- and β-anomers being produced. Although lack of stereospecificity is compensated by the generation of additional analogs (some of the β-anomers have significant activity),[5] the unpredictable stereochemistry of the Koenigs-Knorr method and the need to develop procedures which do not require a large excess of glycosidating reagent have stimulated a search for better methods for stereospecific α-glycosidation. One approach, utilized in the synthesis of the 6'-hydroxy-4'-epi analog of 2a, employs acid-catalyzed condensation of 2b and glycal 109.[5,88] The outcome (56% overall yield, stereospecific) is superior in terms of both yield and stereochemistry to that realized using the corresponding glycosyl halide under Koenigs-Knorr conditions. More recent work indicates, however, that the silver triflate method of Hanessian[89,90] may well have eclipsed earlier procedures. Thus the coupling of 2b with the 4-deoxydaunosamine derivative 110 (1.1 equiv) under the agency of CF_3SO_3Ag provides the corresponding α-glycoside in 70% yield.[91] Similarly, reaction of 111 (α-Cl anomer)



110, R=H 111, R=OCH_3

with 1.5 equiv. of 2b gives the α-glycoside (70%);[92] coupling of 4b and 108 (1.45 equiv.) with CF_3SO_3Ag gives the α-glycoside (46%) which affords 4a (37% overall yield).[5] Additional examples are available.[13,93] The preparations of other analogs are summarized elsewhere.[1,5,94-96]

Conclusions - Efforts toward the synthesis of the anthracyclines have provided a chemical harvest which is already bounteous and continues to be reaped. Overall yields for the total synthesis of the intact, chiral natural products 1a-3a, as well as 4a, 5a and their analogs, are now on the order of 5% in the best cases. Whether total (or partial) synthesis will emerge as the practical solution for the future is, however, a question whose ultimate answer depends on many as yet incompletely resolved factors which include the relative economics of fermentative and total synthesis, the potential therapeutic superiority of non-natural anthracyclines, and future advances in total synthesis. It is the opinion of the author that at the present time fermentation remains the preparative method of choice, but this verdict is much less certain than it would have been five years ago. Five years hence such a judgment may be indefensible. Synthetic chemists would not be distressed.

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Chapter 29. Pharmacophoric Pattern Searching and Receptor Mapping

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Introduction - It has long been believed that a drug is active because of the presence of certain key atoms or functional groupings. Ehrlich[1] called such essential functionality a "pharmacophore", just as functionality imparting color to a compound is termed a chromophore. Ehrlich and others speculated that drugs acted by fitting biological "receptors".[2]

According to drug-receptor theory, a bioactive molecule interacts with a biomolecular receptor to form a complex, thereby eliciting a biological response.[3] It appears that normal chemical intermolecular forces are sufficient to explain drug-receptor interactions and enzyme catalysis without postulating additional, "vital" forces.[4] However, the old "lock and key" picture of rigid drug fitting rigid receptor is giving way to one of flexible but complementary interacting units.[5]

For the purposes of this chapter, we define a drug as any substance, natural or synthetic, which elicits a biological response; and a drug receptor as any biomolecular site at which a drug binds or is otherwise "recognized" in eliciting the biological response. With this broadened definition, a receptor may be a polypeptide binding site on a membrane; an active site of an enzyme; an intercalation or alkylation site in DNA; etc. Whenever a receptor "recognizes" a drug, those structural features of the drug which are "recognized" make up what may be called a pharmacophore. The geometrical arrangement of these structural features is termed a pharmacophoric pattern. The receptor must contain certain features complementary to those of the pharmacophore for the "recognition" to occur; the arrangement of these complementary features is called a receptor map. A recent mathematical model stresses the symmetry of enzyme-substrate recognition; the enzyme "recognizes" a substrate pharmacophoric pattern, just as the substrate "recognizes" a complementary receptor pattern.[6] As an increasing number of drug binding biomolecules are being isolated and characterized, it is perhaps timely to review the utility for drug design of the concepts of pharmacophoric pattern searching and receptor mapping.

Pharmacophoric Patterns - A pharmacophoric pattern has been defined[7] as "a collection of atoms spatially disposed in a manner that elicits a biological response". Such patterns have been classified as topologic (graph-theoretic or connectivity-based substructural fragment) and topographic (geometric, usually 3-dimensional) patterns.[7] Topographic patterns depend on molecular conformation and should be more suitable for describing drug activity - even for chemically dissimilar compounds having the same type of bioactivity.

Topographic pharmacophoric patterns may conveniently be defined in terms of atom types and interatomic distance.[7] Distance geometry has been shown to uniquely define 3-dimensional molecular structures, except for their chirality.[8] Properties of pharmacophoric patterns which have been defined include chirality (stereochemistry),

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orientation (which face of a 3-D pattern is presented to the receptor), accessibility (which atoms in a pattern are accessible to the receptor), and indefiniteness (for example, when a lipophilic group in the drug can engage more than one hydrophilic region on the receptor).[7] Patterns can be made up of atoms of a specific type; of atoms of a general type (e.g., hydrogen-accepting); of atoms with a certain partial charge; of functional groups; and of negative mass (i.e, atoms at a certain position which may interfere with receptor interaction).[7]

Pharmacophoric patterns have been proposed as the result of structure-activity studies, modeling studies, mechanistic studies, and enzyme structural studies. While two atom ("distance") and three atom ("triangle") pharmacophoric patterns have been proposed, we would not expect these to be as discriminating as more detailed patterns. Proposed pharmacophoric patterns have been reviewed by Kier,[9] Korolkovas,[10] and Gund.[7] Some additional proposed patterns of drug pharmacophores and of complementary receptor maps are listed in Table I.

Table I. Some Recently Proposed Pharmacophoric and Receptor Patterns

Bioactivity	Pattern	References
Thyroxine Binding	Receptor Map	11,12
Anti-inflammatory (arylacetic)	Receptor Map	13
Hypnotic (barbiturate)	Receptor Map	14
Insulin Release (sulfonylureas)	Receptor Map	15
Acetylcholinesterase	Receptor Map	16
Analgesic (opiate)	Pharmacophore	17,18,19,20,21,22,23
Antimalarial	Pharmacophore	24
Sweet Taste	Receptor Map	25
Dihydrofolate Reductase Inhibition	Receptor Map	26
α-Adrenergic	Receptor Map	27
Prostaglandin-like	Pharmacophore	28

One would like an automatic procedure for discovering a common pharmacophoric pattern among a group of similarly acting drugs. The statistical techniques of pattern recognition (nearest neighbor, learning machine, etc.) have been used with limited success to correlate biological activity with molecular geometry-derived information such as a generalized X-ray scattering function (molecular transform).[29] However, these techniques appear not yet to have been applied to discovery of pharmacophoric patterns as defined above. Furthermore it may prove difficult to automate the discovery procedure, because of the complicating factors detailed below.

Many bioactive molecules are flexible, and their minimum energy conformation need not correspond to the receptor-bound conformation.[7] Furthermore, conformational dynamics may be important, with receptor molecule and substrate both changing conformation in order to elicit the biological response.[5,30] In some cases, then, a system could show different pharmacophoric patterns for compounds which preferentially bind to the receptor's ground state, its activated state, and an intermediate state (transition state analogs).[4]

It is reasonable to suppose that a drug may be "recognized" by its receptor, at least weakly, in one of its major solution conformations; this has been called "remote recognition of preferred conformation",[31] and "the weak binding approximation".[32,33] A collision theory argument suggests that fastest binding would occur for a molecule reacting from its ground state conformation.[34] However, prudence would dictate that, when looking for pharmacophoric patterns, all low energy conformations - say, those within 6 kcal of the minimum - be considered.[35,36] But this exhaustive approach is infeasible for molecules with many rotatable bonds, where an astronomical number of conformations are possible. In such cases, studying more rigid analogs can narrow the possibilities.[37-40]

Pharmacophoric Pattern Searching - Once a pharmacophoric pattern has been proposed, it would be useful to look for that pattern in other drug molecules - in order to test and refine the pattern; to identify available compounds which should be tested for the bioactivity of interest; and to provide feedback to chemists synthesizing new potential drugs. Such a pattern searching procedure requires a three-dimensional structure for the molecules to be examined, and a method of discovering the presence of the pattern in those molecules.

Crystallographically determined molecular structures have been collected[41] and are available over a dial-up computer network.[42] Other molecules and other conformations may be conveniently generated by the technique of computer-assisted molecular modeling.[7,37,43,44] A prototype computer program to discover occurrences of a given pharmacophoric pattern in molecular structures, MOLPAT, has been described.[7,45] As a test of the program a hypothetical analgesic pharmacophoric pattern, consisting of an aromatic ring and a remote nitrogen atom, was extracted from the crystal coordinates of the prototype analgesic, morphine. A search for that pattern in other molecules disclosed its presence in the crystal structure of LSD, as shown in Fig. 1.[7] LSD does appear to be a mild analgesic.[7] Another reported computer program explores conformations of one structure to find superpositions with another structure.[46]

Beyond Pharmacophoric Patterns - In classical structure-activity studies, medicinal chemists systematically vary a chemical structure and, based on resulting bioactivity, draw conclusions about "essential" structural requirements. More recently, quantitative structure-activity relationships (QSAR's) have allowed a "factoring out" of some of the factors (distribution, lipophilicity, electronic and steric effects) affecting bioactivity.[47,48,49] While such QSAR's tend to give a rather hazy view of receptor geometry, Hansch and others have used indicator variables ("dummy" variables) to "map" receptor sites.[49] Considerations of molecular shape[27,50] and pharmacophoric patterns[7,37] have led to more specific "receptor maps". In further development of this idea Weinstein,[51] among others, has calculated electrostatic potential maps which may express the initial attractive potential between drug and receptor. In another approach, Kier[52] and Weinstein[51] have correlated bioactivity with the calculated interaction energy between a drug and an amino acid residue postulated to occur at the receptor site. Recently, Marshall has developed a computer-assisted

method to superimpose the pharmacophoric patterns of similarly acting drugs and sum their molecular volumes in order to derive an "excluded volume map" of the receptor.[15,37]

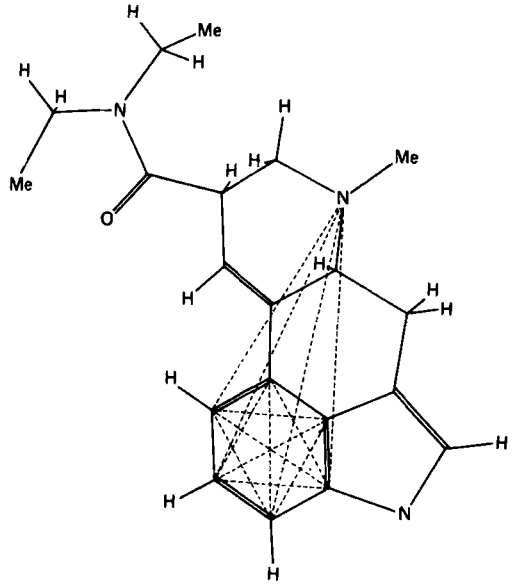


Fig. 1. Presence of an analgesic pharmacophoric pattern discovered in LSD by program MOLPAT (from Ref. 7 with permission).

Even more detailed than a receptor map would be a three-dimensional picture of the receptor. X-ray crystallography of isolated receptors is increasingly offering just such a picture - first at low resolution, where the contoured surfaces resemble the "blob" of the conventional receptor site representation; and finally at atomic resolution, with and without substrate or inhibitor present, so that individual active site residues may be identified and mechanisms of action proposed.[53] It is nevertheless worth stressing that even a crystal structure of the substrate-receptor complex does not explain the dynamics of chemical action, nor does it account for the resultant biological action. Mechanistic speculation based on a crystal structure is still speculation, and will be judged by how well it explains other experimental data.

Recently, high-quality theoretical calculations have been used to explore the energetics of proposed enzyme mechanisms, using enzyme crystal coordinates.[12,54-58] Indeed, simply viewing the enzyme crystal structures and "docking" substrates onto them, using interactive computer graphic techniques,[44] has been shown to afford useful insights.[59,60]

Applications - From a number of proposed pharmacophoric patterns, as detailed in Table I and earlier reviews,[7,9,10] we have chosen three examples where such patterns or receptor maps appear to offer insights. Where possible, the work has been placed in the context of other, related studies.

1. Bacterial Peptide Synthesis Inhibitors - The prokaryotic (bacterial) ribosome, which governs protein synthesis, is structurally different (simpler?) than the eukaryotic ribosomes of its infected host; it is established that many relatively non-toxic antibiotics bind selectively to the former.[61] In the classical model of the mechanism of protein synthesis, peptidyl-tRNA is held on the ribosome at a position ("P-site") adjacent to the position ("A-site") where the attaching aminoacyl-tRNA is bound.[62] Based on the recognition of common topological patterns, Harris and Symons had proposed that several antibiotics act at the "transpeptidase" site (where the peptide bond is formed).[63] Building on this work and a conformational study of lincomycin, Cheney proposed a detailed three-dimensional model for the natural substrates when bound to the transpeptidase site and related the antibacterial activities of lincomycin, chloramphenicol and erythromycin to this model.[64] Hahn and Gund found a different conformation of chloramphenicol which not only matched Cheney's model of the bound natural substrates, but which also could correlate a great deal of structure-activity data for chloramphenicol analogs.[65] However it is still controversial whether chloramphenicol binds to the A-site, the P-site or both.[62]

This model of the active site of transpeptidase has been used for the "rational" design of a novel antibacterial agent. A transition-state analog of the natural substrates inhibits bacterial peptide synthesis *in vitro*.[66]

2. Anti-inflammatory Receptor - In another application, a receptor map was derived for the prostaglandin cyclooxygenase active site, by folding arachidonic acid into a conformation which rationalized its stereospecific oxidative cyclization to the *endo*-peroxide, PGG_2 (Fig. 2a).[13] The map accommodated the fatty acid precursors of the PG_1, PG_2 and PG_3 series, and was consonant with biochemical studies of the enzyme. The same receptor map can be fit by anti-inflammatory arylacetic acids, such as indomethacin (Fig. 2b) and pirprofen, which inhibit the enzyme, and agrees with structure-activity data for these compounds.[13] This map, or a somewhat different map due to Scherrer,[67] may prove useful for deriving new types of anti-inflammatory drugs.

3. Dihydrofolate Reductase Inhibitors - This enzyme (DHFR) reduces dihydrofolic acid (1) to tetrahydrofolic acid - an essential cofactor with important roles in DNA synthesis and cell growth. DHFR can be widely different in structure in different cells, and inhibitors which exploit these differences include clinically useful antibacterial, antiprotozoal, immunosuppressant and antineoplastic drugs. A vast amount of synthetic analog work in this field has been done, much of it by Baker.[68] Hansch and co-workers used data from several groups and QSAR techniques to "map" receptor space for DHFR from rat liver, from

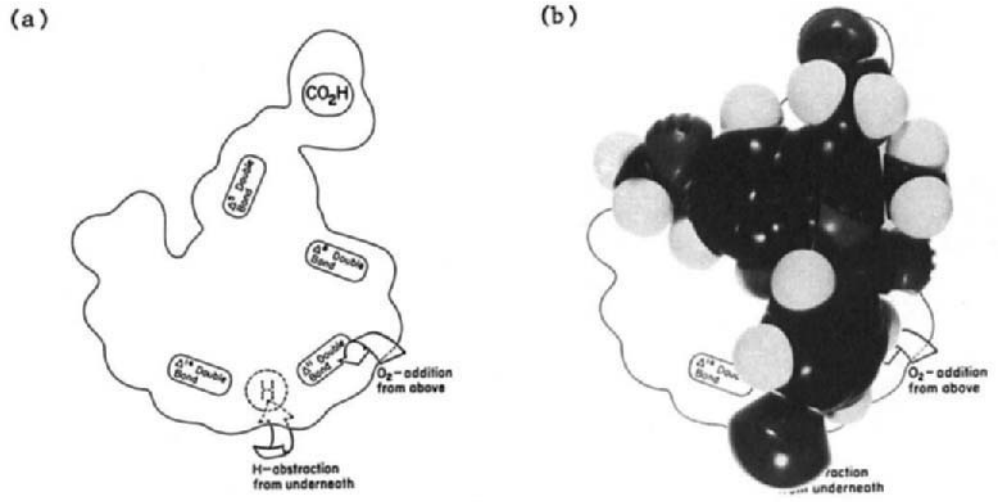
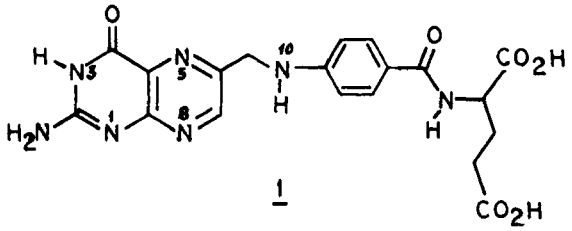
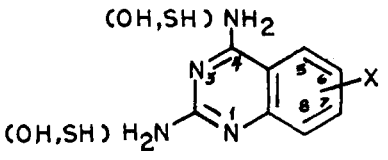


Fig. 2. (a) Hypothetical receptor map of the active site of prostaglandin cyclooxygenase, showing points of binding or reactivity with the enzyme substrate, arachidonic acid. (b) The anti-inflammatory arylacetic acids - here, indomethacin - which inhibit this enzyme, can fit the same receptor map in a way which explains their structure-activity relationships (from Ref. 13 with permission).

bacteria, and (indirectly) from the malarial parasite.[69] For a series of 67 quinazoline derivatives (2) inhibiting Streptococcus faecium, regression analysis suggested that 4-OH or 4-SH binds much less



1



2

effectively than 4-NH_2; 5-substituents bind to the enzyme hydrophobically but large bulk is not tolerated; and 6-substituents are open to solvent (whereas they are not when binding mammalian enzyme). Simon derived a comparable receptor map of DHFR by a "minimal topological differences" technique.[26]

The strongly inhibiting drug methotrexate differs from the enzyme's substrate, dihydrofolic acid, by exchanging the tautomeric 4-keto group by 4-amino, and by having a methyl group on N-10. Spectral studies[70] and CNDO/2 calculations of model structures[71] suggested that 1 is protonated more strongly at N-1. A calorimetric study supports protonation of methotrexate on binding to DHFR, but 1 apparently is not protonated until the reduction step.[72]

Recently there has been a resurgence of interest in this well-studied system, due to the crystal structure determination of methotrexate bound to Escherichia coli DHFR,[73] and of methotrexate and nicotinamide adenine dinucleotide phosphate reduced form (NADPH) cofactor bound to Lactobacillus casei DHFR.[74,75] Figure 3 shows a stereo drawing of the α-carbon atoms of L. casei DHFR, with the methotrexate inhibitor and NADPH cofactor in place. The methotrexate is draped over a pocket in the middle of the enzyme, with the glutamate chain on the right, the p-aminobenzoyl group horizontal to the viewer in the center, and the pteridine group angling deeply into the enzyme's interior. The cofactor has the dihydronicotinamide group just to the left of the pteridine moiety, dramatically poised to deliver a hydride equivalent during reduction of substrate; the phosphonucleotide tail of the cofactor curls around to the left and upwards. The stage is set for correlating the structure-activity data from hundreds of synthetic inhibitors with specific interactions on the enzyme. The task will not be easy. Complications include amino acid sequence variation in DHFR from different species; binding of cofactor; and altered positioning of different inhibitors. There is already evidence that the pteridine ring in methotrexate may be "turned over" with respect to its conformation in dihydrofolic acid (1) during reduction.[74-76] Nevertheless the enzyme structure "provides an opportunity for rational design of a new class of DHFR inhibitors that would incorporate elements of both substrate and cofactor in a single molecule".[75]

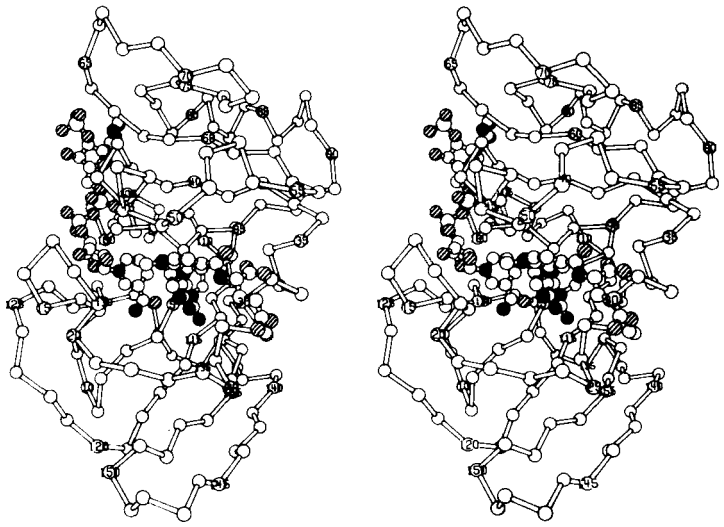


Fig. 3. Stereo drawing of α-carbon atoms of L. casei DHFR enzyme with bound inhibitor (methotrexate: right center of view) and cofactor (NADPH: left center). Stereo viewers are recommended, but many people can see the 3-D image by placing an index card between the images and staring, one eye on each image, until the images coalesce. The bound substrate and cofactor have oxygen atoms shaded and nitrogen atoms blackened, respectively. Every fifth residue is numbered (from Ref. 75 with permission).

4. Other Examples - The design of an antihypertensive agent based on a hypothetical model of the active site of angiotensin-converting enzyme is now a well-known story.[77,78] A study of binding of thyroxine to prealbumin utilized the crystal coordinates of the protein.[11] Inhibitors of the oxygen binding site of hemoglobin were derived as an exercise in rational drug design.[79] There are a large number of proposals of how the conformationally flexible enkephalins could cause analgesia by mimicking the opiates.[17-23]

Conclusion - Drug research is gradually moving away from broad empirical screening, toward "rational" design of inhibitors/activators of target enzymes/receptors. Drug design methods which explicitely consider the 3-dimensional structure of drug and/or receptor would seem to deserve a place in the medicinal chemist's armamentarium.

Pharmacophoric pattern searching attempts to identify the key geometric features of a drug structure that cause it to be "recognized" by its "receptor". Postulating specific interaction points on the receptor for attachment of the drug leads to a "receptor map". Both pattern and map lead to testable hypotheses, and may be used to design novel structures which should mimic a known drug or inhibit a target enzyme.

Like any other "rational" approach to the complex problem of drug design, these methods are fraught with difficulties. A compound "predicted" to be active may prove to be inactive for any of the usual reasons - distribution, elimination, metabolism, competitive binding, etc. Nevertheless, this approach can bring a new dimension to the chemist's perception of structure-activity relationships and, especially in combination with other techniques, may point the way to novel and effective drugs.

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Chapter 30. Pharmacokinetics and Drug Design

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Introduction - Pharmacokinetics as a scientific discipline has undergone rapid development over the past decade. The mathematics and applications of pharmacokinetics are well described in text-books.[1-3] Utilization of pharmacokinetic concepts and models to aid in the individualization of dosage regimen in patients with different disease states is now commonplace.[4,5] The impact of pharmacokinetics on drug design, however, is just beginning to be realized. A recent symposium focused on the approaches used in the design of biopharmaceutical properties through prodrugs and analogs.[6]

Pharmacokinetic input in drug design recognizes the fact that, for some drugs, lack of optimum or desirable therapeutic activity is not due to an inadequacy in drug-receptor site interaction, but rather due to an inappropriate concentration and/or time-course of drug presentation to the receptor. For example, an oral anti-inflammatory agent may require a rather high oral dose for therapeutic activity in spite of a low ED_{50} when introduced intravenously. A synthetic program aimed at the preparation of improved derivatives may benefit greatly from comparative pharmacokinetic data obtained after oral and parenteral dosing, although parenteral use of the drug in humans may not be anticipated. Data obtained from properly designed pharmacokinetic experiments may suggest the reason(s) why a high oral dose is required, e.g. slow absorption, poor oral absorption, or extensive first-pass metabolism.

The appreciation that structural modification can greatly alter drug pharmacokinetics is certainly not new. The barbiturates present classic examples of how minor structural changes can significantly alter their distribution and elimination characteristics,[7] giving rise to a spectrum of compounds with varying degrees of onset and duration of activity. What is perhaps novel and exciting is a growing willingness on the part of investigators to probe the relationship between pharmacokinetic parameters and structure. The state of the art is of course far from what has been achieved in quantitative structure activity relationships (QSAR). At present, no unified theory has been developed which would permit prediction of pharmacokinetic behavior, even a qualitative one, based solely on structural considerations. However, there have been numerous examples in the past few years in which considerable improvement of pharmacokinetic characteristics have been obtained through structural modification. Collation of these data may eventually allow for comparison, and (dare we dream) prediction, of pharmacokinetic parameters based on structure. In the present chapter, we review recent developments which focused on comparative pharmacokinetics of structurally similar compounds. The pharmacokinetic phenomena discussed here will be categorized into (i) absorption, (ii) distribution, and (iii) elimination.

Absorption - Much of the effort in synthetic medicinal chemistry involves attempts to confer oral activity to parenterally active drugs. Lack of oral activity due to inadequate concentrations in the systemic circulation

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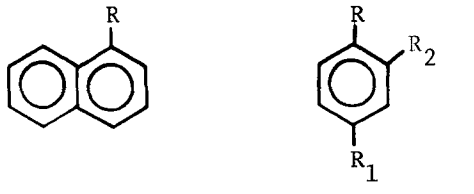
may be the result of (i) gastrointestinal drug degradation, (ii) poor absorption, or (iii) extensive metabolism during the first passage of drug through the gut and the liver. An interesting example of the interactions of these factors was provided by the absorption of sodium γ-hydroxybutyrate (GHB), and its lactone analog, butyrolactone (GBL).[8,9,10] GHB is an active intravenous anesthetic in man and has been suggested as a potentially useful anti-parkinsonian agent because of its ability to increase brain dopamine concentrations when injected into animals. Its oral activity at low doses is poor because of substantial hepatic metabolism during absorption.[8] However, even when oral doses are increased to the extent where first-pass metabolism is saturated, oral hypnotic activity is still not realized.[9] Pharmacokinetic experiments revealed that at these high doses, the bioavailability of GHB was close to unity but the peak plasma concentrations did not increase proportionately with dose. Apparently, GHB transport across the small intestine became capacity-limited with increasing dose and the rate of drug absorption decreased to such an extent that threshold hypnotic plasma concentrations were not achieved, even with very large oral doses.

GBL was found to hydrolyse rapidly in plasma, with a half-life of approximately 1 minute. In spite of this rapid biotransformation, oral activity of GBL was almost identical to that achieved after intravenous administration of GHB.[10] Apparently, GBL is not subjected to any of the processes which decrease peak plasma concentrations, viz: gastrointestinal degradation, capacity-limited transport and first-pass metabolism. The use of lactone analogs as a general approach to improve the bioavailability and/or rate of absorption of other hydroxy-acids, e.g. prostaglandins, has, however, not been tested.

Drugs containing the catechol and phenol functional groups present interesting examples of how absorption and metabolism can be altered through minor structural modification. These groups are vulnerable to conjugation, possibly both in the gut and in liver, and esterification of them could substantially decrease the extent of such metabolic inactivation. Minatoya[11] showed that bitolterol, the di-p-toluate ester of N-tert-butylarterenol, gave good bronchodilator activity with a more prolonged duration of action than its parent compound or isoproterenol when administered intraduodenally. More interestingly, bitolterol appeared to distribute preferentially to lung tissues as compared to plasma or heart, thereby eliciting the bronchodilator action while simultaneously reducing undesirable cardiovascular effects. The slow *in vivo* hydrolysis of bitolterol apparently allowed distribution to tissues before its biotransformation, leading to a beneficial selectivity in its pharmacological activity. Bodor *et al*[12] prepared pro-drugs of L-dopa with attachments on the three metabolically sensitive centers of the molecules, viz: the carboxylic acid, the amino and catechol groups. Plasma concentrations of L-dopa and dopamine were obtained in dogs after oral administration of these derivatives, which included carboxylic acid esters, phenol esters, amides, peptides and various combinations of these functions. Although some of these derivatives could increase the bioavailability of L-dopa by 2.5-fold, the effect was less than the 5-fold reduction in L-dopa dose requirement which could be brought about via co-administration of the decarboxylase inhibitor, L-α-methyl dopa hydrazine. More dramatic results were obtained with esters of epinephrine[13,14] and adrenalone[15] synthesized

for improved glaucoma therapy. Wai et al[14] showed that the dipivalyl ester of epinephrine was about 10 times better absorbed than epinephrine, most probably due to its increased lipophilicity.

Some of the β-adrenergic receptor blocking drugs also show considerable first-pass metabolism.[16] For example, the bioavailability of oral propranolol (1) is low and is subjected to wide inter-subject variations. A major metabolite formed, 4-hydroxy-propranolol, is also an active β-blocker. Garceau et al[17] found that the hemissuccinate ester of 1 gave considerably higher and less variable oral absorption in dogs than a corresponding dose of 1. It was suggested that blocking of the hydroxy groups in 1 reduced O-glucuronidation during first-pass metabolism. Examination of the bioavailability of other β-blockers, together with that of 1, (Table I), showed that blocking of the position para to the amino alcohol side chain, such as in 4, 5, 6 and 8, leads to substantial increases in oral bioavailability. Substitution of the naphthyl nucleus by an indole ring, as in 9, also favorably affects bioavailability. Ortho substitutions, such as in 2, 3 and 7, do not increase bioavailability significantly.



1

2, 3, 4, 5, 6

R = $-OCH_2CH(OH)CH_2NHCH(CH_3)_2$

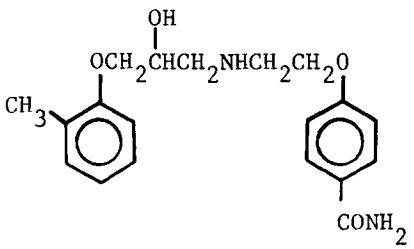
2: R_1 = H, R_2 = $-CH_2CH=CH_2$

3: R_1 = H, R_2 = $-OCH_2CH=CH_2$

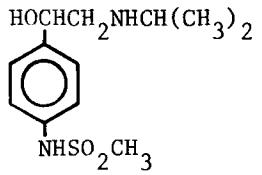
4: R_1 = $-CH_2CONH_2$, R_2 = -H

5: R_1 = $-CH_2CH_2OCH_3$, R_2 = -H

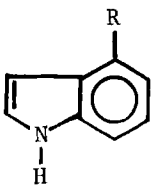
6: R_1 = $-NHCOCH_3$, R_2 = -H



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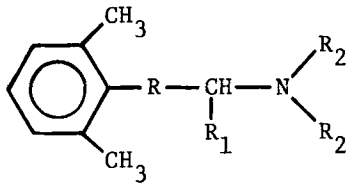


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Table I: Comparative bioavailability of β-blockers.[16]

	Compound	Bioavailability (% of dose)	Dose-dependent Bioavailability
alprenolol	2	≃ 10	Yes
propranolol	1	≃ 30	Yes
tolamolol	7	≃ 30	-
oxprenolol	3	24-60	No
atenolol	4	⩾ 40	No
metoprolol	5	≃ 50	No
sotalol	8	⩾ 60	-
pindolol	9	≃ 100	No
practolol	6	≃ 100	No

The antiarrhythmic drug lidocaine (10) is also subjected to extensive first-pass metabolism. Its metabolites, monoethylglycinexylidide and glycinexylidide, are pharmacologically active. The former shows antiarrhythmic and convulsive properties and is a potent emetic agent in animals,whereas the latter potentiates the convulsive effects of both lidocaine and monoethylglycinexylidide.[18] Tocainide (11) however, is apparently not subjected to first-pass metabolism. Mexiletine (12) also has a high oral bioavailability. Both 11 and 12 possess a methyl group alpha to the amino nitrogen.



10: R = -NHCO-, R_1=H, R_2=C_2H_5

11: R = -NHCO-, R_1=CH_3, R_2=H

12: R = -OCH_2-, R_1=CH_3, R_2=H

Considerable success has been obtained in preparing orally active derivatives of penicillins. Many of these compounds are plasma-labile esters, e.g. pivampicillin and bacampicillin, but a non-labile analog such as amoxycillin can also substantially increase absorption. Recently, lactonyl esters of ampicillin and other penicillins,[19] and acyloxymethyl esters of a cephalosporin[20] were prepared and found to have superior oral absorption characteristics compared to their respective parent compounds.

Modification of drug absorption rate can also lead to improvement of therapeutic efficacy. This is usually accomplished by altering the dissolution characteristics of the drug via formulation techniques rather than through structural modification. A recent attempt of the latter approach involves the preparation of 4-β-methyl digoxin, which showed improved absorption over that of digoxin in humans,[21,22] but not greater cardiac activity.[21] A stoichiometric complex was also used to increase digoxin absorption rate. Higuchi and Ikeda[23] prepared a hydroquinone complex of digoxin which is more readily soluble than digoxin itself. Bioavailability testing in humans showed that this complex yielded faster digoxin absorption than that provided by a commercial tablet. Decrease in absorption rate, however, is desirable in the case of theophylline because of the necessity for frequent dosing due to its relatively short elimination half-life and narrow therapeutic index. Bodor *et al*[25] prepared a series of 7-acyl and 7, 7'-acylditheophylline derivatives. These authors suggested that 7, 7'-succinyldtheophylline may have the desirable dissolution characteristics for it to be a useful controlled release form of theophylline *in vivo*.

Improvement of drug solubility may also be necessary because an injectable form of the drug is desired. Bourne *et al*[26] showed that the antitumor compound acronine can be transformed into the soluble and labile acetylacroninium salts which may be suitable for intravenous use.

Absorption kinetics are an important consideration for drugs which produce toxicity at the absorption site. Decrease in gastrointestinal irritations of acetylsalicylic acid was accomplished via an analog diflusinal, which is 2',4'-difluoro-4-hydroxy-3-biphenylcarboxylic acid[27]. This compound apparently emerged after pharmacologic screening

of several hundred analogues of salicyclic acid, and is said to possess increased analgesic and anti-inflammatory activity. Triglycerides containing aspirin in place of one or more fatty acid residues of the molecule have also been synthesized. The 1, 3-di-fatty acyl-2-aspirin glyceride appeared to be partially absorbed through the rat intestine; it did not produce ulceration in the rat stomach.

Distribution - The extent of distribution of a drug to extravascular areas can be represented pharmacokinetically either by measurement of tissue drug concentrations in laboratory animals, or by calculation of the apparent volume of distribution (V_d). For those drugs that display mono-exponential decay, V_d is a proportionality factor relating the drug concentration in serum or plasma to the amount of drug in the body. A large V_d implies that the amount of drug in the body is extensively distributed outside the plasma space.

Most drugs, however, exhibit multi-exponential delay and are fitted to a two-compartment model. In such cases, V_d may be calculated by several methods[1] producing such volume terms as $V_d(\beta)$, V_d(area), V_d (extrapolated) and V_d (steady state). These volumes often do not have the same values. However, each can have meaningful application as described in detail elsewhere.[1]

The extent of distribution of a drug to the extravascular space is influenced by: (i) the degree of ionization of the drug, (ii) lipophilicity and tissue lipid content, (iii) plasma protein and tissue binding, (iv) tissue perfusion, and (v) specialized transport processes. The relationships between partition coefficient, ionization, and V_d have been examined and mathematical expressions derived for predicting V_d based on these physicochemical properties.[29] The effects of binding on V_d have also been discussed in a recent review[30] and described quantitatively by Gibaldi and McNamara[31]. V_d is related to the free fraction of drug in plasma (f_p), free fraction of drug in the tissues (f_T), volumes of plasma (V_p) and the tissue compartment (V_T), in the following manner:

$$V_d = V_p + \frac{f_p}{f_T} V_T$$

In general, only non-ionized, free drug is available for tissue distribution and the extent of tissue distribution is related to lipid solubility.

In the design of new drugs, the utility of estimating V_d is that it might afford a qualitative estimation of drug concentration at the site of action, whether the effect is related to concentration in plasma, as for warfarin,[32] or to tissue concentrations as with β-methyl-digoxin and its metabolite digoxin.[33] For example, administration of the methoxymethyl ester of hetacillin resulted in more extensive tissue distribution of ampicillin than when ampicillin itself was given.[34] This could be predicted on the basis of the reported V_d values of ampicillin and the hetacillin ester which are approximately 30% and 85% of body weight, respectively. Decreased tissue distribution of adriamycin was observed when it was administered as DNA-complex.[35] Thus, structural modification for selective drug distribution is a potential avenue for enhancing efficacy.

The two classes of pharmaceuticals which best exemplify this rationale for drug design are the antimicrobial and antitumor antibiotics. In considering the distribution of the penicillins and cephalosporins in the body, the very important role of plasma protein binding must be recognized. Both of these groups of antimicrobials display a wide span in the extent of drug bound to plasma proteins, ranging from 20 to 96% for the penicillins and 6 to 96% for the cephalosporins. Accordingly, the apparent volumes of distribution of these drugs have been correlated with both percent binding and lipophilicity.[36] Binding characteristics must also be considered in interpreting in vitro determinations of minimum inhibitory concentrations, as only the unbound drug is biologically active.[37]

Because of the variable binding and lipid solubility characteristics, the penicillins and cephalosporins also exhibit varying degrees of distribution into tissue sites. Drug penetration to the infective site is a major factor in selection of the antibiotic of choice and should also be a major factor in design of new drugs. The value of pharmacokinetics in such situations can be illustrated with the results of several recent investigations of the distribution of cephalosporins into interstitial fluid. Tan and Salstrom,[38] using a skin window technique, concluded that penetration of human interstitial fluid by cefazolin was poor compared to cephalothin and cefamandole. However, Carbon et al[39] found that cefazolin was superior to cefamandole and cephaloridine in penetrating rabbit interstitial fluid using tissue cages. In addition to the species and model differences in these two studies, only the latter was done following multiple dosing. These differences could be resolved by calculation of the appropriate pharmacokinetic parameters. Bergan has made such calculations for cefazolin and several other cephalosporins in humans and found cefazolin to be the drug of choice in distributing to tissue fluids.[40] This conclusion was based on the apparent volume of tissue compartment, calculated from V_d (steady state) and a comparison of the magnitudes of the transfer rate constants k_{12} (entry into tissue) and k_{21} (exit from tissue). Of the cephalosporins studied, only cefazolin had a k_{12}/k_{21} ratio greater than unity, apparently indicating that cephazolin entry into tissues is more rapid than its exit. It should be mentioned, however, that in view of the variations in lipid content and binding properties of different tissues, such calculations may have somewhat limited usefulness if information regarding the distribution into a particular tissue is desired.

Recent advances in the field of physiological pharmacokinetics, in which organ blood flows, volumes, and drug clearances are considered, have been useful in estimating drug concentrations in a specific organ. These models have been applied for the most part to antitumor agents. For example, such a model has been used to show which anticancer agents might be most effective in chemotherapy of ovarian cancer via peritoneal drug administration.[41]

One method that has been used to improve selective localization of chemotherapeutic agents is via enzymatic drug activation.[42] This involves conversion of the inactive parent compound to the active drug selectively at the site of action. In the course of developing anticancer agents to localize in the prostate gland, derivatives of colchicine were made as

substrates specific for prostatic acid phosphatase, the activating enzyme which converts the prodrug to colchicine.[43] A similar mechanism for selective distribution has been proposed for delivery of radiolabeled glucose to the brain and heart for use as imaging agents, with trapping of the isotope in these tissues being reflective of glucose utilization.[44]

In addition to the possibility of improving therapeutic effects, structural modifications utilizing pharmacokinetic analysis of drug distribution can explain and potentially avoid toxicities by selective distribution away from specific tissues. This has been demonstrated in relation to aminoglycoside nephrotoxicity,[45] and accumulation of β-adrenoceptor antagonists in the lungs.[46] However, the full potential for the use of pharmacokinetic distribution volumes in drug development has yet to be realized.

Elimination - Elimination is defined here as excretion and/or metabolism of intact drug from plasma. In general, the main excretion routes are renal and biliary, and the predominant metabolic route is hepatic. The pharmacokinetic parameters most widely used to characterize plasma concentration decay are the apparent first-order rate constant (K), elimination half-life ($t_{1/2}$), and clearance (Cl). Clearance is defined as the volume of plasma removed of drug in unit time and can be compared with physiologic organ perfusion rates. For drugs with mono-exponential decay, $Cl = V_d \cdot K$. When more than one route of elimination exists, the sum of individual clearances equals the total plasma clearance:

$$Cl_{total} = Cl_{metabolic} + Cl_{renal} + Cl_{biliary} + Cl_{others}$$

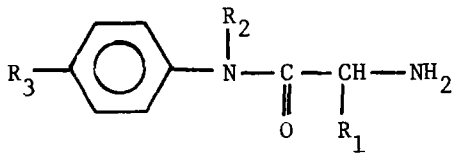
Structural modifications may affect one or more routes of clearance, thereby creating a net effect on total plasma clearance.

In drug design, alterations in elimination rate are often desired for drugs which are cleared too rapidly in the body. When metabolic clearance is predominant, examination of the relative amounts of metabolites formed may identify the labile or reactive functional groups which need to be modified or protected to decrease elimination. The prostaglandins, for example, generally have a short duration of action due to rapid metabolism and excretion. The main metabolic enzymes involved are the 15-hydroxyl dehydrogenase and β-oxidative systems. A compound that is not a substrate for these systems and yet retains prostaglandin activity is obviously desirable. Recently, for example, cis-Δ^4-15(S)-methyl prostaglandin $F_{1\alpha}$ was found to meet these criteria.[47] After oral administration of the methyl ester of this prostaglandin in the rat,[48] urine was void of the 15-keto metabolite and showed low levels of dinor and intact C-20 compounds, suggesting that the dehydrogenase system was not operative for the analog, and that β-oxidation was substantially reduced. In contrast to other prostaglandins, intact parent compound could be recovered in urine. Prolongation of analgesic activity of methadone has also been attempted by replacing the metabolically labile C-6 dimethylamino group with a piperidinospiro function.[49] Frincke *et al*[50] gave oral doses at about 0.2 mg/kg of the ketone, alcohol and acetate forms of the piperidinospiro derivative to mice and found the duration of analgesia to be 3.0, 6.5, 20.5 hr, respectively. In contrast, oral methadone at a dose of 22.5 mg/kg only gave a duration of analgesia of 8.0 hr.

A recent report described an interesting approach with which a marked increase in the duration of activity of the enzymes carboxypeptidase G and arginase was effected through conjugation with soluble dextran.[51] As measured by enzyme activity in the blood, the native enzymes had respective half-lives of 3.5 and 1.4 hours. When coupled with dextran, the half-lives of these two enzymes in blood rose dramatically to 17 and 12 hours, respectively. Attempts to prolong drug activity through incorporation of an alkylating group on the drug has been investigated. The reactive group presumably binds with a nucleophile at or near the receptor site, thereby increasing drug residence time. Kornet et al[52] studied derivatives of succinimides with alkylating groups at the 2-position and found the most striking increases in the duration of anticonvulsant effect with the α-bromoamide and maleimidoethyl analogs. Both these analogs have a longer duration of effect (> 4 hours) than the currently available phensuximide and methsuximide. The toxicity of these alkylating derivatives, however, is an obvious concern.

The relationships between structure, renal clearance and total body clearance of a series of substituted xylidines were examined by Berlin-Wahler et al[53] after intravenous infusion of these compounds in dogs (Table II).

Table II: Mean renal and total body clearance of substituted xylidines in dogs.



Compound #	R_1	R_2	R_3	Renal Clearance (ml/min)	Total Body Clearance (ml/min)
11	CH_3	H	H	14	48
13	H	H	H	19	55
14	C_2H_5	H	H	6	161
15	H	CH_3	H	28	188
16	CH_3	CH_3	H	45	172
17	H	C_2H_5	H	16	218
18	CH_3	C_2H_5	H	8	260
19	CH_3	H	C_3H_7O	3	128
20	H	C_2H_5	C_3H_7O	2	301

The data suggested that renal clearance of these compounds was relatively unimportant. Alkyl substitution at the amide nitrogen apparently increased total body clearance while similar substitution alpha to the amino nitrogen did not significantly alter this parameter. Introduction of a p-OC_3H_7 moiety, 19 and 20, caused substantial increases in total body clearance when compared to tocainide (11).

Prodrug formation has been a well-tested approach to increase duration of activity of various drugs. A prodrug can be designed such that the rate of conversion to its parent compound becomes rate-limiting. This would result in an increase in the apparent elimination half-life

of the drug. Drugs that possess phenolic or alcoholic hydroxyl groups usually exhibit short duration of action because of their hydrophilicity and their susceptibility to attack by conjugating enzymes. For esters of terbutaline and apomorphine,[55] increasing steric hindrance in the acyl moiety was found to lower the in vitro hydrolysis rate in the presence of serum esterases. When injected into mice, the di-isobutyrl apomorphine derivative[56] gave detectable brain apomorphine concentrations for six hours compared to only 90 minutes when apomorphine is administered. Increase in the duration of action was also observed with the phosphate ester of Δ^8-THC[57] and the disteroidyl enol esters of 17-β-estradiol.[58] In these compounds, covalent bonds were formed at the metabolic site, so that cleavage of the prodrug must occur before metabolism can take place. However, inhibition of metabolism can also be effected through structure modification at neighboring non-metabolic locations. For example, formation of the 5'-valerate ester of 9-β-D arabinofuranosyladenine (ara-A)[59,60] apparently made the derivative resistant to deamination. Additionally, this ester served as a competitive inhibitor of ara-A.[59]

Drug enantiomers may differ pharmacokinetically as well as pharmacologically. In man, R(+) warfarin showed a significantly longer elimination half-life than the S(-) enantiomer (35 vs. 24 hours)[61] and in rats the same trend was seen for the congener phenprocoumon.[62] Significant, though less drastic, pharmacokinetic differences were seen after administration of racemic amphetamine[63] and fenfluramine.[64] In both cases, there was significantly more d-enantiomer in the brain and plasma of rats after intraperitoneal injection. Thus, choice of a particular enantiomer for clinical use may be based on pharmacokinetic, as well as pharmacologic, grounds.

Toxicity differences in a series of analogs may be related to their relative elimination rates. Netilmicin, a new aminoglycoside antibiotic, has been shown to be less nephrotoxic than gentamicin in rats.[65] Pharmacokinetic analysis in man reveals that these compounds have similar half-lives, while netilmicin shows more extensive extravascular distribution and therefore, a faster plasma clearance.[66] Similarly, metformin, a biguanide anti-diabetic, is less toxic than phenformin reportedly because of its shorter half-life (1.5 vs. ~ 12 hours).[67] When a compound causes direct organ tissue damage the toxicity is more easily assessed. The analgesics acetaminophen and phenacetin both appear to cause their hepatotoxicity via oxidation of the amide nitrogens. Nelson et al[68] reasoned that this reaction could be blocked by N-methylation in these compounds. Indeed, N-methylacetaminophen and N-methylphenacetin were found to have much reduced hepatotoxicity.

Summary - It has been shown that pharmacokinetic parameters can be favorably affected by structural modification without, in certain cases, alteration in the pharmacological properties of the parent drug. Detailed pharmacokinetic information obtained for lead compounds may provide a valuable guide as to how subsequent syntheses should be directed.

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Chapter 31. Metals in Treatment of Disease

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Introduction - Inorganic materials, metals and metallic salts have been used in medicine since the dawn of time. Metallic ions have been shown to play vital roles in many biological processes. However, research in developing new metal containing molecules as therapeutic agents has been overshadowed by the vast amount of attention directed to the design of organic molecules with novel biological activity. Nevertheless, in recent years certain metals have been associated with specific therapeutic benefit. Research in the field of bioinorganic chemistry is making increasingly rapid progress with the help of new sophisticated instrumentation and analytical methodology. Earlier work in the use of metals and metal complexes in biology and medicine has been bibliographically described by Ellis.[1] The therapeutic use of different metallic compounds as well as the benefit or detriment of their interaction with organic molecules has been reviewed.[2] Cleare[3] reported on the therapeutic use of metal complexes with special emphasis on the cancer chemotherapeutic utility of platinum complexes. This review will attempt to identify newer metallo-compounds which appear to owe their biological properties to the metal contained therein as well as the status of older ones which have attracted special attention. Since there is little if any structure-activity relationship between the different metals, they will be discussed in the order of their appearance in the periodic table.

Group Ia Lithium - Lithium salts were used indiscriminately over the early part of this century for treatment of gout, epilepsy, insomnia, hypertension and as salt substitutes in cardiac disease.[4] Unfortunately, toxicity associated with lithium salt therapy was not known at that time. Many cases of serious side effects and deaths resulted which led to their disuse. In recent years judicious use of lithium salts, primarily lithium carbonate, for the control of manic symptoms has reestablished their utility in clinical practice.

Mania (affective disturbances) recently has been divided into two functional groups, bipolar and unipolar affective disorders. Bipolar disorders include both manic and depressive episodes whereas unipolar disorders show only recurrent depressive episodes. Patients with bipolar disorders usually have an early onset of the illness (20-35 years age) with high incidence of mania and suicide. Some evidence suggests that this condition is hereditary, transmitted through the X-chromosone. On the other hand, onset of unipolar disorders usually occurs at a later age (30-45) and hereditary association is less well defined.

Recent extensive reviews[4-6] describe current indications for lithium therapy primarily in bipolar disorders and experimentally in unipolar depression as well as schizo-affective schizophrenia, alcoholism, premenstrual cramps and character disorders. These reports also call attention to the narrow therapeutic index associated with its use and the need for careful monitering of serum levels. Serum levels of 0.6 to 1.5 mEq per liter are usually sufficient for management of symptoms. A dose of 300mg of lithium carbonate t.i.d. or q.i.d. is recommended to maintain these

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levels and slow release formulations have been reported to be advantageous dosage forms.[7] Levels in excess of 1.5 mEq often produce toxic side effects leading to hypothyroidism and at times permanent neurologic damage.[8] Lithium also can induce nephrogenic diabetes insipidus by inhibition of antidiuretic hormone (ADH), which gradually disappears when lithium is discontinued.[9] Although the mechanism of beneficial action of lithium carbonate in manic conditions is not well understood, symptom changes have been correlated with cerebral-evoked potentials and EEG changes during treatment.[10] Stabilization of dopaminergic receptor sensitivity in the nigrastriatal dopamine pathway has been invoked as contributing to its effect on manic-depressive episodes.[11]

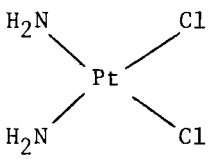
Transition Elements - Group VIII-Iron - Current therapy for controlling abnormally high body iron content was reviewed in vol 13 of this series.[12] Iron deficiency anemia remains frequent in many populations including the affluent countries where dietary deficiencies are not prevalent. Iron homeostatis is unique in that, unlike other trace metals, it is regulated primarily by absorption and not by excretion. Recent reports detail the mechanism of iron absorption across the intestinal mucosa in the lumen of the small intestine to receptors in the brush border of the mucosal cells and into the cell, followed by transportion of cellular iron into the plasma in combination with various chelating carriers.[13,14] Ferrous sulfate remains the cheapest form of supplemental iron, although numerous pharmaceutical forms are available.[15,16] Parenteral administration of iron salts offers little or no advantage over oral administration and is associated with anaphylactic side effect potential as well as the risk of sarcoma development at the site of injection.[17] However, intravenous administration of dextran iron complex ("Imferon") has been reported to produce a beneficial rise in hemoglobin in patients undergoing regular hemodialysis.[18] Although ferric salts are not used therapeutically, ferric ion participates in many biochemical events. Physicochemical aspects of iron metabolism and contribution of the Fe(II) - Fe(III) redox couple, involving a one electron transfer, have been well described. Aisen[19] reported evidence that free radical intermediates, resulting from this redox, may account in part for the toxicity of iron in iron overload. Inonophore A23187 has been reported[20] to facilitate transport of Fe(II) across lipid membranes. Although toxicity of A23187 precludes its utilization in any therapeutic form, the potential exists for a non-toxic ionophore with these properties.

Alksne and Smith[21] described the novel use of an iron acrylic compound for the stereotaxic thrombosis of intracranial aneurysms in patients who were poor risk candidates for surgery. The compound polymerized rapidly, did not fragment and was non-toxic. Good results were reported in experimental animals and humans.

Osmium - Surgical removal of the synovial membrane (synovectomy) in the affected joints of rheumatoid arthritis (RA) patients is one means of giving relief to, as well as slowing the crippling process in patients with early active RA. Osmium tetroxide injected into the affected joints has been used since 1952 in Europe to perform a synovectomy by chemical means (synoviothesis). Neither procedure gives lasting results. However, the latter can be carried out easily with only a few days hospitalization

and can be repeated without risk. Recent controlled studies support the benefit of chemical synovectomy.[22,23] The clinical significance of the accompanying superficial damage to articular cartilage has not been determined although the results have not shown harmful late complications.[24] In contrast
knee joint of rabbits with antigen induced arthritis.[25]

Platinum - Intensive studies of different transition metal complexes in cancer chemotherapy have focused attention on platinum complexes as being of greatest potential.[26-32] Cis-platinumdiaminodichloride (PDD, NSC-119875, "Platinol", cisplatin) has been approved recently for treatment of testicular and ovarian cancer as well as showing promise in other neoplastic conditions.[33] Its application in therapy is limited by renal, hematologic and ototoxicity.[34,35] The major limiting toxic effect is renal toxicity which appears to be dose related, ranging from transient elevation of blood urea nitrogen and serum creatinine to irreversible renal insufficiency. Similar toxicity is displayed by mercury and other heavy metals. Mannitol-induced diuresis, previously used to lessen acute renal toxicity produced by mercury, has likewise been shown to reduce the nephrotoxicity of cisplatin both experimentally[34] and clinically.[36] Prehydration and concomitant osmotic diuresis improved the therapeutic index of cisplatin allowing for the administration of larger doses (3mg/kg/iv) which produced encouraging clinical responses in testicular carcinoma, carcinoma of the head and neck and ovarian carcinoma with reduced limiting side effects. Comparative renal pathological studies confirmed that mannitol-induced diuresis considerably decreased the renal toxicity associated with cisplatin therapy.[37] Flameless atomic absorption spectrometry has been reported[38] to be a convenient and sensitive method for the analysis of platinium in biological material.



Although the mechanism of antineoplastic action of cisplatin has not been defined, physicochemical studies have shown an interaction with nucleosides, nucleotides, homopolynucleotides and DNA. Cisplatin has been reported to exhibit little binding affinity for thymidine and greater affinity for guanosine, adenosine and cytidine.[39] Interaction of cytidine, thymidine, adenosine and guanosine with cisplatin, measured by uv difference spectroscopy, led the authors to suggest that cisplatin bound selectively to GC base-pairs or GC rich regions of DNA as compared to AT regions. Other workers[40] using transmission electron microscopy in conjunction with x-ray probe microanalysis have shown that HeLa cells treated in vitro with cisplatin concentrate platinum on the inner side of the nuclear double membrane and in the nucleolus, indicating that cell death may be due to Pt interference at the initiation sites of DNA synthesis. Cisplatin also was reported to decrease the level of testosterone-dependent esterases in kidneys of experimental animals.[41] The association between this induced decrease in testosterone levels and testicular antitumor activity has not been answered. A related compound cis-platinum-(3,4-diaminotoluene)dichloride produced profound changes in the permeability of rat liver mitochondria. Phosphate transport into the mitochondria was inhibited irreversibly. Magnesium and calcium ions were rapidly released. Stimulation of mitochondrial respiration, similar to the apparent uncoupling of

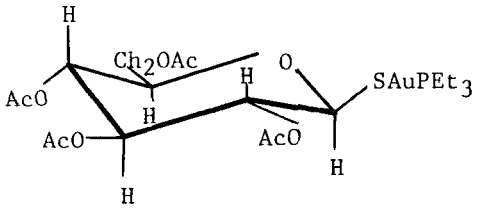
oxidative phosphorylation, was observed. Association of these membrane effects with antitumor activity is being studied further.[42]

Group Ib Copper - Copper in the metallic state, as well as different salt forms, has been used since antiquity for the treatment of different inflammatory conditions.[43] A copper complex of salicylic acid ("Permalon") has been reported to be of clinical benefit in RA when administered intravenously.[44] Copper chelates of a number of non-steroidal antiinflammatory agents (AI) were reported to be more potent and less ulcerogenic than the AI agents themselves.[45,46] Copper ions *per se* have been reported to afford protection against inflammation in some cases without the assistance of other agents.[47] It has been suggested that the AI drugs exert their beneficial effects by facilitating the release of cupric ions from serum albumin either by a direct competitive complexing mechanism or through a remote mechanism whereby the drug becomes bound to a site remote from the copper ion and facilitates its release by allosteric effects.[48-51] The beneficial effects of copper salts have been questioned because of conflicting results obtained in rat and guinea pig models of inflammation.[52] A computer study has identified the critical chemical feature required in an AI agent to exploit serum albumin bound copper.[53] Although the mechanism of action of copper complexes is unknown, stimulation of superoxide dismutase has been proposed as a contributing factor.[54]

Gold - Monovalent gold, stabilized as a coordinated salt, was first used for the treatment of RA by Lande[55] as a result of an assumed relationship between RA and tuberculosis. Forestier's[56,57] early recognition of this benefit established chrysotherapy as a form of treatment for RA. Since that time the benefit of its use was controversial, until the Empire Rheumatism Council[58] in 1960 confirmed the efficacy of gold therapy for RA in a controlled study. In Japan,[59] as well as elsewhere,[60] gold therapy has been reported to be of special benefit in the treatment of allergic bronchial asthma. Although this therapeutic regimen has not been developed to its fullest clinical utility, recent publications[61,62] state that gold preparations inhibit the release of the mediators of immediate hypersensitivity which are concerned with allergic responses. Gold sodium thiomalate ("Myochrysine") and gold thioglucose ("Solganol") are two of the most commonly used salt forms. Numerous recent reports describe conflicting results when treating arthritic patients with gold preparations.[63,64] Although the drugs must be administered intramuscularly for therapeutic effect, a direct correlation between clinical response or toxicity with blood gold levels has not been observed using standard 50mg weekly drug injections.[65] Adjustment of individual dosage schedules to maintain a serum gold level >300 μg per cent was reported to optimize the therapeutic response.[66] Gold therapy has been reported to have a very high toxicity liability. However, careful reevaluation of the statistics leading to that conclusion as well as major changes in use of the drugs and careful laboratory monitoring of treated patients, has allowed its increased use with a reduced likelihood of toxic reactions.[67] The exact mechanism whereby gold(I) compounds exert their beneficial effect is not known but numerous activities have been observed (antibacterial activity, inhibition of lysosomal enzymes, stabilization of collagen, inactivation of collagenase, immunological mediation, prostaglandin synthesis inhibition) as being involved in bringing about their therapeutic benefit.[68-71] The

biochemistry and pharmacology of gold compounds have been reviewed in detail.[72,73] Reports on gold tissue distribution usually focus on its binding with plasma or serum albumin. However patients undergoing chrysotherapy, accumulate gold in organs of the reticuloendothelial system, such as the liver. Danpure reported that rats concentrated gold in the lysosomes of liver cells.[74] In contrast to plasma albumin binding, gold in lysosomal supernatants was bound to molecules of a wide molecular weight range. Shaw has studied distribution of gold in rat kidney cortex cells.[75] Unlike cadmium or mercury, gold does not induce a binding protein such as metallothionein. Involvement of glutathione as a nonprotein thiol binding component is undergoing further study.

Experimental gold(I) compounds in which the gold has a coordination number of two in contrast to those in clinical use with a coordination number of one produced antiarthritic activity in adjuvant induced arthritis in rats when administered by the oral route.[76] Gold sodium thiomalate was effective only when administered parenterally.[77] Auranofin [SK&F D-39162, (2,3,4,6-tetraacetyl-1-thio-β-D-glycopyranosato-5)(triethylphosphine)gold], a member of this group, was reported to exhibit a unique pharmacological profile when compared to gold sodium thiomalate in a variety of systems.[77] Intramuscular administration of gold sodium thiomalate appeared to be less effective in suppressing the lesions of adjuvant induced arthritis in rats, produced relatively higher levels of gold in the sera and kidneys and marked toxicity when compared with an equivalent amount of auranofin administered orally. Auranofin inhibited in vitro lysosomal enzyme release from both animal[78] and human[79] leukocytes at concentrations equivalent to those obtained in the blood of patients on clinical trial. Its suppression of lymphocyte function[80] as well as antitumor potential has been reported.[81] Clinical trials and animal studies indicate that auranofin may prove to be a more effective and less toxic form of chrysotherapy for RA than currently used agents.[82-84]



Group 2b Zinc - Much use of zinc sulfate in therapy is a result of the recent recognition that zinc deficiency in man is associated with a variety of toxic phenomena. Differential zinc content in plasma, serum, erythrocytes hair, urine and saliva has been used as an index of deficiency. However, a correlation of zinc deficiencies with specific clinical symptoms has not been unequivocally established. Thus responsiveness to zinc therapy is the most reliable index of zinc deficiency. Recent advances in analytical methods have greatly facilitated measurements of zinc at the metabolic level. The average concentration of zinc in plasma when assayed by modern techniques is approximately 100 μg/dl.[85] Zinc deficiency in infants and preadolescent children leads to growth retardation, impaired learning and failure of sexual maturation. Other associated abnormalities are impaired taste and/or smell and poor wound healing.[86] Low zinc levels in the sera of patients with liver disorders is associated with high urine levels.[87] It has been proposed that during acute stress an inflammatory response activates phagocytic cells to release a leukocytic endogenous mediator (LEM) which acts on the liver to increase the movement of zinc from plasma to hepatocytes, thus producing an apparent systemic hypo-

zincemia.[85] However, benefit of zinc therapy in this situation has not been determined. Supplemental zinc therapy in malnourished children has been shown to stimulate growth of the thymus glands.[88] It is recommended therapy in post surgical patients and patients with major burns to facilitate wound healing. Zinc sulfate supplementation has been used to treat recurrent idiopathic oral ulcerations (canker sores) where it was most effective in those persons with serum zinc levels $\leq$ 100 µg/dl.[89] The benefit of oral zinc sulfate in the treatment of acne was not increased when combined with Vitamin A.[90] Prophylactic use of oral zinc as a therapeutic adjunct, in contrast to a trace metal supplement, reduced the frequency of crisis experienced by patients with sickle cell anemia.[91,92] This was not a double blind study. A reduction in the percentage of irreversibly sickled red blood cells was observed during treatment. The authors suggested that zinc produced its effect by increasing the filterability of partially deoxygenated sickled cells, allowing egress of excess calcium ion. Accumulation of calcium in red blood cells has been associated with the sickling phenomena and damage to the red cell membrane. Uremic impotence in a group of chronic hemodialyzed males was reversed by zinc sulfate treatment.[93] Zinc salts have been recommended as chemotherapeutic agents for animals infected with foot and mouth disease virus.[94] These salts were shown to inhibit production of foot and mouth disease virus in tissue culture by interfering with the cleavage of high-molecular-weight precursors of FMDV-specific proteins. Local application of silver-zinc-allantoinate proved to be a convenient and effective regimen for the treatment of chronic cutaneous leg ulcers in patients who failed to respond to other therapeutic agents, including antibiotics.[95]

Group 5b Vanadium - The observed potent inhibition of (Na,K)-activated renal ATPase by sodium orthovanadate (Na_3VO_4) prompted investigation of its natriuretic and diuretic properties.[96,97] Intravenous injection of Na_3VO_4 (1.5 µmol) in rats resulted in a prompt and dramatic increase in urine flow and sodium excretion. The action was reversible and the animal model responsive to a second dose. The authors concluded that vanadate was a potent natriuretic diuretic with as yet unknown therapeutic utility.

Radioactive Isotopes - Radioactive isotopes of metals are widely used in medical therapy and diagnosis. A tabulated review references this area in detail.[98]

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